Colonization of cattle by non-O157 Shiga Toxin-producing *Escherichia coli* serotypes

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ABSTRACT

Shiga toxin-producing *E. coli* (STEC) is an important food- and water-borne pathogen of humans, causing Hemorrhagic Colitis and Haemolytic Uremic Syndrome. Colonization of both cattle and human hosts is mediated through the action of effector molecules secreted via a type III secretion system (T3SS), which forms attaching and effacing lesions (A/E). The necessary effectors which form A/E by manipulation of host signalling and actin nucleation are present on a pathogenicity island called the Locus of Enterocyte Effacement (LEE).

It has been reported that vaccination of cattle with Type III-secreted proteins (T3SPs) from STEC O157 resulted in decreased shedding. In order to extend this to non-O157 STEC serotypes, we examined the serological cross-reactivity of T3SPs of serotypes O26:H11, O103:H2, O111:NM and O157:H7. Groups of cattle were vaccinated with T3SPs produced from each of the serotypes and the magnitude and specificity of the responses were measured resulting in limited cross reactivity. Overall, results suggest that vaccination of cattle with T3SPs as a means of reducing the risk of STEC transmission to humans will induce protection that is serotype specific.

To pursue the possibility of a cross-protective vaccine, we investigated the protective properties of a chimeric Tir protein against STEC serotypes. Several studies have reported that Tir is highly immunogenic and capable of producing high antibody titers. Potter and colleagues also demonstrated that the vaccination of cattle with Δ*tir* STEC O157 strain did not protect as well as the wildtype strain. We constructed thirty-mer peptides to the entire STEC O157 Tir protein, as well as to the intimin binding domain of the Tir protein from STEC serotype O26, O103 and O111. Using sera raised against STEC O157 and non-O157 T3SPs, we identified a number of immunogenic peptides containing epitopes unique to a particular serotype. Two different chimeric Tir proteins were constructed containing the STEC O157 Tir protein fused with six STEC non-O157 peptides with or without the Leukotoxin produced by *Mannheimia haemolytica*. However, the vaccination of mice with the chimeric protein did not protect against challenge with STEC O157 or STEC O111. These results suggest that to achieve

cross protection against STEC serotypes using a recombinant protein vaccine, other immunogenic and protective antigens must also be included.

In order to identify other immunogenic and cross-protective antigens we cloned and expressed the genes coding for 66 effectors and purified each as histidine-tagged proteins. These included 37 LEE-encoded proteins and 29 non-LEE effectors. The serological response against each protein was measured by Western blot analysis and an enzyme-linked immunosorbent assay (ELISA) using sera from rabbits immunized with T3SPs from four STEC serotypes, experimentally infected cattle and human sera from 6 HUS patients. A total of 20 proteins were recognized by at least one of the STEC T3SP-vaccinated rabbits using Western blots. Sera from experimentally infected cattle and HUS patients were tested using an ELISA against each of the proteins. Tir, EspB, EspD, EspA and NleA were recognized by the majority of the samples tested. Overall, proteins such as Tir, EspB, EspD, NleA and EspA were highly immunogenic for both vaccinated and naturally infected subjects.

Based on the above results, two different mixtures of secreted proteins (5 proteins and 9 proteins) were used to vaccinate mice and test the level of shedding following challenge with STEC O157. Overall, the cocktail vaccine containing 9 immunogenic effectors including Tir, EspB, EspD, NleA and EspA was capable of reducing shedding as effectively as the current STEC T3SPs vaccine, Econiche®.

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DEDICATION

For my grandfather Sergio,

That his love for science continued through the generations….

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ABBREVIATIONS USED

A/E	Attaching and effacing
AI	Autoinducer
ANOVA	One-way analysis of variance
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BCIP	5-Bromo-4-chro-3-indolyl phosphate
CDC	Centre for Disease Control and Prevention
CDT	Cytolethal distending toxin
CesT	Chaperone for *E. coli* secretion of Tir
CFIA	Canadian Food Inspection Agency
CFU	Colony forming unit
Cif	Cycle inhibiting factor
CpG	Cytosine-phosphate-guanosine
DNA	Deoxyribonucleic acid
DC	Dendritic cell
EAST1	Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1
E. coli	*Escherichia coli*
Efa1	Factor for adherence
EHEC	Enterohemorragic *E. Coli*
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPEC	Enteropathogenic *E. coli*
ER	Endoplasmic reticulum
Esp	*E. coli* secreted protein
GALT	Gastrointestinal-associated lymphoid tissues
Gb3	Globotriasol ceramide
Gb4	Globotetraosyl ceramide
GEF	Guanine nucleotide exchange factors
GM-CSF	Granulocyte macrophage-colony stimulating factor
GMP	Guanosine monophosphate

GST	Glutathione S-transferase
H antigen	Flagellar antigen
Hep-2 cells	Human epidermoid cancer cells
IEC	Intestinal epithelial cell
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IL	Interleukin
IL-1R	Interleukin-1 receptor
JAK-STAT-IRF1	Janus-activated kinase-signal transducer and activator of transcription-interferon regulatory factor 1
IHF	Integration host factor
K antigen	Capsular antigen
kDa	Kilodalton
LB	Luria-Bertani
LEE	Locus of enterocyte effacement
Ler	LEE-encoded regulator
Lpf	Long polar fimbriae
LP	Lamina propria
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTA	Lipoteichoic acid
HDP	Host defense peptide
H-NS	Histone-like nucleoid structuring protein
HUS	Hemolytic uremic syndrome
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
Map	Mitochondrial associated protein
M cell	Microfold cell
MIA	Thrombotic microangiopathy
MIP	Macrophage inflammatory protein
MLN	Mediastinal lymph node

NBT	Nitroblue tetrazolium
NE	Norepinephrine
NF-κB	Nuclear factor-kappa B
Nle	Non-LEE effector
NM	Non-motile
NO	Nitric oxide
N-WASP	Neural Wiskott-Aldrich syndrome protein
O antigen	Somatic antigen
OI	O island
OmpA	Outer membrane protein A
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PG	Peptidoglycan
PI	A blood group glycolipid antigen
PI3K	Phosphatidylinositol 3-kinase
PNPP	Diethanolamine phosphate
PRR	Pattern recognition receptor
QS	Quorum sensing
RBC	Red blood cell
RNA	Ribonucleic acid
ROCK	Rho-associated coiled coil-containing kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
RTX toxin	Repeats in the structural toxin
Saa	STEC autoagglutinaiting adhesion
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPI	*Salmonella* pathogenicity island
SOB	Super Optimal Broth
SRS	Silencer regulatory sequence
STEC	Shiga toxin-producing *E. coli*

Stx	Shiga toxin
THP1	Human acute monocytic leukemia cell line
TIR	Toll/IL-1 receptor
Tir	Translocated intimin receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
TTP	Thrombotic thrombocytopenic purpura
T3SS	Type III Secretion System
T3SP	Type III Secreted protein
USA	United States of America
UCAC	University Council on Animal Care
VTEC	Verotoxigenic *E. coli*
VWF	Von Willebrand factor

1.0 LITERATURE REVIEW

1.1 *Escherichia coli*

1.1.1 Nomenclature

Escherichia coli (*E. coli*) is a Gram-negative, non-sporulating, facultative anaerobic member of the family *Enterobacteriaceae*. This bacterium is approximately 2 μm long, with a diameter of 5 μm, and a cell volume of 0.6-0.7 μm^3 (Kubitschek 1990). Its primary environment is the gastrointestinal tract of mammals and birds, and in humans it remains the predominant facultative anaerobe of the colonic flora (Karmali, Petric et al. 2004). The optimal growth temperature for *E. coli* is 37ºC, but some laboratory strains can multiply at temperatures as high as 49ºC (Fotadar, Zaveloff et al. 2005). This organism, which is also found in soil and water, is commonly transmitted by fecal contamination. *Escherichia coli* colonize the gastrointestinal tract of infants in the first hours of life, where a mutual relationship quickly develops between the bacterium and host. This organism tends to be harmless in the intestinal lumen but in an immunosuppressed host, or when gastrointestinal barriers are broken, even non-pathogenic strains can cause infection.

Serological typing of *E. coli* is based on the characterization of its dominant O (lipopolysaccharide), K (capsular) and H (flagellar) antigens (Sojka 1965; Gyles 1994; Sussman 1997). The H antigen represents the distinctive serological epitopes located on the flagellin protein, which are responsible for the formation of the flagella. To date, 53 different H antigens have been identified in *E. coli* (Sojka 1965). The K antigen represents a class of thermolabile polysaccharides that form sheaths, envelopes, or capsules. This antigen was first identified through bacterial agglutination tests. The somatic O antigen represents the cell wall lipopolysaccharide complex, and over 170 different O antigens have been identified (Gyles 1994). This antigen is able to remain active at high temperatures ranging from 100°C to 121°C (Sojka 1965). The combination of the O and H antigen defines the serotype, and over 700 different *E. coli* serotypes have been recognized to date. Some of these serotypes are pathogenic and are defined by their virulence characteristics.

Shiga toxin-producing *E. coli* (STEC), also known as Enterohemorrhagic *E. coli* (EHEC) or Verotoxigenic *E. coli* (VTEC), is a zoonotic pathogen that causes bloody diarrhea and hemorrhagic colitis in humans (Thorpe, Hurley et al. 1999; Tuttle, Gomez et al. 1999; Karmali, Petric et al. 2004). This organism was first recognized as a pathogen in 1982, after two outbreaks of hemorrhagic colitis and abdominal cramps among individuals who ate undercooked hamburgers at a fast food restaurant (Riley, Remis et al. 1983). The Centre for Disease Control and Prevention (CDC) isolated an *E. coli* O157:H7 strain from these infected individuals, and it was soon discovered that this class of *E. coli* expressed a set of toxins called Shiga toxins (Stx) (Riley, Remis et al. 1983). These enterotoxins were isolated from fecal samples, and were found to be responsible for the development of hemolytic-uremic syndrome (HUS) (Karmali, Steele et al. 1983).

1.1.2 Shiga toxin-producing *E. coli* O157:H7

Shiga toxin-producing *E. coli* O157:H7 is the most common STEC serotype, initially described in 1977 by Konowalchuk and colleagues (Konowalchuk, Speirs et al. 1977). This serotype has been the cause of numerous infections worldwide, where at least 30 countries in 6 different continents have reported outbreaks (Nataro and Kaper 1998; Yoon and Hovde 2008). In North America, STEC O157:H7 is the most dominant serotype associated with hemorrhagic colitis and HUS. In the United States alone, this serotype is responsible for 73,480 cases, 2,168 hospitalizations and 61 deaths annually (Mead, Slutsker et al. 1999). Annual losses in medical care are understood to range from 300 to 700 million dollars, where the elderly and the young suffer the most severe manifestations of the disease (Paton, Ratcliff et al. 1996; Tuttle, Gomez et al. 1999; Yoon and Hovde 2008).

Shiga toxin-producing *E. coli* O157:H7 has also been responsible for the most severe STEC outbreaks reported worldwide. In Canada, the largest occurred in Walkerton, Ontario when the water system became contaminated with STEC O157:H7 through fecal matter, resulting in 2,100 cases and 7 deaths (Holme 2003). The biggest European STEC outbreak occurred in Scotland, where meat purchased from a single butcher shop infected 501 people resulting in 20 deaths (Dundas, Todd et al. 2001). The largest Asian outbreak ever recorded occurred in Japan, where an STEC O157:H7

outbreak related to white radish sprouts was responsible for thousands of school children becoming ill (Watanabe, Wada et al. 1996). A number of large outbreaks have also been reported in Africa, which resulted in a significant number of cases and fatalities (Cunin, Tedjouka et al. 1999; Effler, Isaacson et al. 2001).

Pathogenic STEC serotypes share biological characteristics that set them apart from non-pathogenic *E. coli* strains. An important feature which defines STEC O157:H7 from other STEC strains is its inability to ferment D-sorbitol, whereas roughly 75% to 94% of all *E. coli* serotypes ferment sorbitol (March and Ratnam 1986; Nataro and Kaper 1998). This allows for rapid identification by plating on sorbitol-MacConkey medium. Another important characteristic of STEC O157:H7 is its inability to produce β-glucuronidase. Upon hydrolysis, β-glucuronidase can be detected by the use of 4-methyl-umbelliferyl-D-glucuronide through fluorescence (Thompson, Hodge et al. 1990).

Several important differences were revealed when the genome sequences of STEC O157:H7 and *E. coli* K12, a common laboratory strain, were compared. Both genomes share approximately 4.1Mb of a homologous sequence, while the STEC O157:H7 sequence contains an additional 1.34Mb of genomic Deoxyribonucleic acid (DNA) made up of 1387 genes, not present within the *E. coli* K12 genome (Perna, Plunkett et al. 2001). These additional genes are found in 177 O-islands scattered throughout the STEC O157:H7 chromosome. However, the STEC genome is missing a region of approximately 0.53Mb genomic DNA found on the *E. coli* K12 genome. It is supposed that throughout its evolution, STEC O157 acquired large amounts of foreign DNA through lateral gene transfer. This uniquely acquired DNA contains virulence factors, alternative metabolic capacities and numerous prophages. Through the study of phylogenetic trees, STEC O157 has been suggested to have evolved from the less pathogenic *E. coli* O55:H7 serotype (Whittam, Wachsmuth et al. 1988).

1.1.3. Non-O157 serotypes

Over the last fifteen years, non-O157 serotypes have emerged as important enteric pathogens. In fact, over a decade ago, only 50% of USA clinical labs would run diagnostics on stool samples for STEC serotypes other than STEC O157:H7 (Tarr and Neill 1996). Non-O157 serotypes are responsible for sporadic outbreaks both in North

America and in other continents. In the USA alone, these serotypes cause 36,740 illnesses, 1,084 hospitalizations and 30 deaths annually (Mead, Slutsker et al. 1999). Depending on the region, non-O157 strains are responsible for up to 50% of all STEC outbreaks, while in Europe, non-O157 illnesses are more common than STEC O157 (Park, Gates et al. 1996; Brooks, Sowers et al. 2005). Overall, the reported cases of non-O157 infections are likely to be an understatement of the actual prevalence due to the inability of clinical isolation and identification.

An extensive study completed by Brooks and colleagues, analyzed 940 human non-O157 STEC isolates collected by the CDC between 1983 and 2000 (Brooks, Sowers et al. 2005). This study reported that the most common non-O157 serotypes were STEC O26, O111, O103, O121, O45 and O145, where O111, O26 and O103 accounted for over 50% of all isolates. Numerous outbreaks in countries such as Japan, Argentina, Chile, Germany, Australia, USA and Ireland have been attributed to non-O157 infections (Nataro and Kaper 1998; Bettelheim 2007). In countries such as Argentina, non-O157 strains are responsible for the majority of outbreaks and HUS cases reported (Rivero, Padola et al. 2004).

The clinical isolation and identification of non-O157 serotypes is challenging compared to STEC O157. Identification of STEC O157 is relatively simple because of its inability to ferment D-sorbitol (Nataro and Kaper 1998). However, non-O157 serotypes and most non-pathogenic *E. coli* strains ferment D-sorbitol. The current identification method involves the detection of toxin genes such as Stx present in STEC serotypes. Another explanation why non-O157 serotypes are not often reported is because many laboratories do not have the facilities to isolate, identify and characterize these strains (Bettelheim 2007). Interestingly, the increase in identified non-O157 outbreaks over the last few years could be related to better and stricter testing, regardless if a sample is positive for STEC O157:H7, since co-infections can occur with multiple STEC serotypes.

1.1.4 Human diseases

1.1.4.1 Diarrhea and hemorrhagic colitis

Human infections by STEC produce either no symptoms, watery diarrhea, or watery diarrhea involving speckled or grossly bloody stools (Figure 1.1). The bloody watery diarrhea is known as hemorrhagic colitis, and involves feverless cramping, abdominal pain and vomiting (Cleary 2004). Chronic diarrhea is extremely rare with STEC infections, while common diarrhea can last over a week. Fever is only found in a small fraction of patients infected with STEC. Patients who develop fever usually also develop HUS at some stage of hemorrhagic colitis. Several complications can arise from STEC inflammation of the gastrointestinal tract. These complications range from gangrene with peritonitis and sepsis, to rectal prolapse, coma, hemiplegia, pancreatitis and seizures (Lopez, Devoto et al. 1989; Nataro and Kaper 1998; Cleary 2004).

Shiga toxin-producing *E. coli* can also produce a number of toxins and secreted proteins which induce watery diarrhea in humans. During digestion, cells secrete fluid into the lumen with the help of chloride channels. These channels are activated via receptors such as guanylate cyclase C, which cause a change in intracellular second messenger levels (Uzzau and Fasano 2000). Heat stable enterotoxins, such as EAST1, initiate diarrhea by activating the cyclic guanosine monophosphate (GMP) pathway that phosphorylates the chloride channels on the apical membranes of intestinal epithelial cells (IECs). This phosphorylation stimulates chloride secretion, and the inhibition of sodium chloride absorption by the villus tips of cells. This event leads to an increase in luminal ion content, causing water to pull through the paracellular pathway, resulting in osmotic diarrhea (Nataro and Kaper 1998). Shiga toxins are involved in diarrhea by killing absorptive villus tips on epithelial cells, resulting in an imbalance in intestinal absorption and secretion (Kandel, Donohue-Rolfe et al. 1989; Nataro and Kaper 1998). The bloody diarrhea observed with STEC is attributed to the role of Stx on endothelial cells, thrombotic microangiopathy, and the lesions on small blood vessels in the gut (Ray and Liu 2001).

Several secreted proteins play an important role in the production of diarrhea. For example, EspF disrupts the host intestinal barrier function, by inducing the redistribution

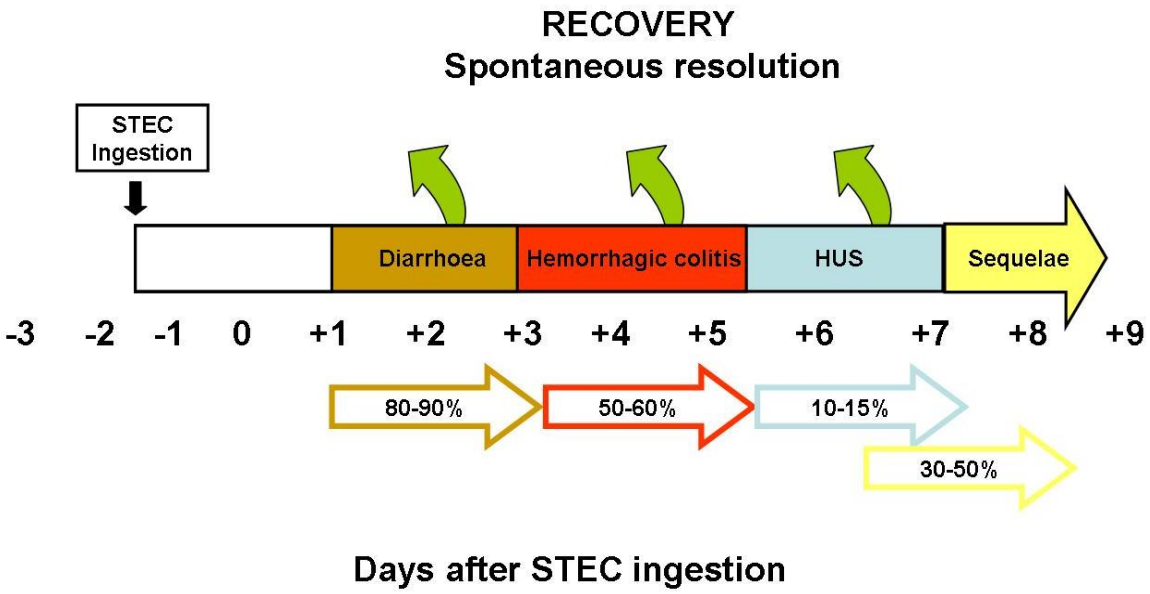


Figure 1.1 The pathophysiology of STEC infection. The onset of diarrhea occurs within the first 3 to 8 days, which can result in hemorrhagic colitis and complications such as HUS. Percentages represent patient likelihood of developing infection (diarrhea, hemorrhagic colitis, HUS and sequelae). Numbers (-3, -2, -1, *etc*…) represent days during infection Sequelae can range from central nervous system involvement, skeletal and myocardial involvement, renal disease, and mortality. Diagram was adapted and modified from (Scheiring, Andreoli et al. 2008).

of the protein occludin which is associated with the stability of tight junctions (McNamara, Koutsouris et al. 2001). This effect results in a loss of trans-epithelial electrical resistance and paracellular permeability, leading to fluid secretion. Other effectors such as EspG, and Map, also affect intestinal barrier function by causing loss of epithelial electrical resistance or by altering mitochondrial function (Dean and Kenny 2004; Matsuzawa, Kuwae et al. 2005). The secretion of effectors causes an up-regulation in the host inflammatory response, by activating NF-κB, which leads to the production of IL-8 and the recruitment of neutrophils (Kaper, Nataro et al. 2004). The activation of NF-κB also causes an increase in the galanin-1 receptor expressed by the epithelial cells in the gut, resulting in excessive fluid secretion.

1.1.4.2 Hemolytic-Uremic Syndrome

Hemolytic-uremic syndrome was first described in 1955 by von Gasser and colleagues in a study of five children with small vessel renal thrombosis and non-immune haemolytic anaemia (Gasser, Gautier et al. 1955). In 1965, a study by Barnard and Kibel hypothesized that HUS could be a result of enteric *E. coli* infections, without the knowledge of the existence of STEC (Barnard and Kibel 1965). Ten years later, Kaplan and colleagues reported the identification of HUS in a group of 83 siblings within 41 families, and concluded that the clustering of cases in endemic areas was a result of an environmental infectious agent (Kaplan, Chesney et al. 1975). It was not until 1983 that Karmali and colleagues were able to identify an *E. coli* strain in the stool of HUS patients that produced an enterotoxin lethal to cultured Vero cells (African monkey kidney cells) (Karmali, Steele et al. 1983; Karmali, Petric et al. 1985). Soon after, it was reported that this toxin was identical to Stx produced by *Shigella dysenteriae* serotype 1 (O'Brien, Lively et al. 1983). These initial studies led to the discovery that Stxs, along with virulence factors produced by STEC, were the main factors involved in the development of HUS.

Typically, HUS occurs most frequently in the summer months and in children younger than 5 years of age (Ostroff, Kobayashi et al. 1989). The increase of summer cases correlates with reservoir shedding which is reported to increase during warm summer months (Chapman, Siddons et al. 1997). An in depth 20 year population study of

USA HUS occurrence in children under the age of 18 found an annual incidence ranging between 0.2 to 3.4 per 100,000 children (Siegler, Pavia et al. 1994). In Canada, annual incidences average 3.1 per 100,000 children under the age of five, while in Europe, countries such as Germany, Italy and Austria report annual incidences of HUS averaging 0.71 in 100,000 children under the age of 15, and up to 2 per 100,000 in children under the age of five (Rowe, Orrbine et al. 1998; Gerber, Karch et al. 2002). However, the highest number of worldwide HUS cases is found in Argentina where 420 new cases are recorded each year with an annual incidence of 12.2 per 100,000 children under the age of five (Rivero, Padola et al. 2004). Overall, it is suggested that 83% of all worldwide HUS cases involving diarrhea are due to STEC infections (Amirlak and Amirlak 2006). Recently, a new study reported that of all STEC O157 infections, 15% will eventually progress to HUS (Figure 1.1) (Tarr, Gordon et al. 2005). This disease is the most common cause of acute renal failure in infants and young children worldwide.

A typical case of HUS begins with an incubation period averaging between 3 to 8 days. Soon after, a patient will develop watery diarrhea which can lead to hemorrhagic colitis with abdominal cramps. The involvement of bloody diarrhea in the gastrointestinal region can result in transmural necrosis with perforation and development of colonic stricture (Siegler 1994). Hemorrhagic colitis occurs in 90% of all HUS cases, which usually triggers the patient, or family members of the patient, to seek medical attention. On average, 50% of cases involve nausea and vomiting while 30% will involve fever (Scheiring, Andreoli et al. 2008). These symptoms lead to nephrological and hematological changes, where patients undergo thrombocytopenia, hemolytic anemia and renal failure. The haemolysis observed with HUS is a result of damage to erythrocytes in the small blood vessels. Although the main organs involved in HUS are the kidneys and the gastrointestinal area, other regions such as the pancreatic, skeletal, myocardial and the central nervous system can also be affected. Pancreatic involvement, indicated by glucose intolerance, edema and the elevation of pancreatic enzymes occurs in less than 10% of cases (Andreoli and Bergstein 1982). The involvement of the central nervous system during HUS was found to develop in up to 25% of cases and can lead to irritability, lethargy and seizures. Rarely, central nervous system involvement leads to cerebral edema and coma (Amirlak and Amirlak 2006; Scheiring, Andreoli et al. 2008). Skeletal

and myocardial involvement can also develop, however this is extremely unusual (Askiti, Hendrickson et al. 2004). The majority of children who develop HUS will have some level of renal deficiency. Roughly two thirds of HUS patients will require dialysis therapy, while one third will have lesser renal involvement and dialysis will not be required (Gerber, Karch et al. 2002).

Mortality rates in HUS patients range between 3-5% during acute phase, which usually involve renal disease and severe central nervous system (CNS) involvement (Amirlak and Amirlak 2006). Of the patients that developed HUS, 20-30% develop extra-renal events, 20% of recovered patients show end-stage renal disease while 60% show signs of renal failure during acute phase. Several risk factors such as the administration of antibiotics, bloody diarrhea, elevated serum leukocyte count, fever, being under the age of ten, female gender and the use of antimotility agents, are believed to increase the susceptibility of developing HUS (Wong, Jelacic et al. 2000; Siegler and Oakes 2005).

1.1.4.3 Thrombotic thrombocytopenic purpura

Thrombotic microangiopathy (MIA) is distinguished by microvascular thrombosis, thrombocytopenia, microangiopathic hemolytic enemia and multi-organ injury. Thrombotic MIA is comprised of two syndromes called thrombotic thrombocytopenic purpura (TTP) and HUS, and both develop by separate mechanisms. These variants cause thrombocytopenia, microangiopathic hemolytic anemia, fever, renal abnormalities and neurological effects (Zheng and Sadler 2008). Early studies suggested that STEC O157 was responsible for the development of TTP. A case study in 1986 reported a 53 year old woman with bloody diarrhea, microangiopathic hemolytic enemia, neurological effects and fever consistent with TTP symptoms (1986). A later study in 1990 reported similar symptoms where STEC O157 was isolated from stool samples (Kovacs, Roddy et al. 1990). Reported cases in adults involving neurological dysfunction were often diagnosed as TTP, while cases in children involving glomerular damage were diagnosed as HUS.

It was not until the late 1990s that it was discovered that TTP patients suffer from a severe deficiency of a plasma metalloprotease called ADAMTS13 that cleaves von Willebrand factor (VWF), or an ADAMTS13 deficiency due to autoantibodies which

inhibit its activity or clear it from circulation (Furlan and Lammle 1998). Thrombotic thrombocytopenic purpura patients that do not produce this protease have large amounts of VWF agglutinated to circulating platelets in areas of intravascular shear stress (Chow, Turner et al. 1998). Although rare, Stx's can impair ADAMTS13 cleavage of VWF multimers which results in damage to endothelial cells, and may induce renal microvascular thrombosis (Nolasco, Turner et al. 2005). Of TTP cases tested, 45% to 100% of patients had reduced ADAMTS13 activity, while HUS patients rarely or simply did not demonstrate a deficiency in ADAMTS13 activity (Vesely, George et al. 2003).

Thrombotic thrombocytopenic purpura is a rare disease with an incident rate of 0.3 to 1 per 100,000 yearly in the USA (Torok, Holman et al. 1995). Before the introduction of plasma exchange, 90% of TTP patients perished. However, plasma exchange has reduced mortality to between 8 to 15%, making it a curable disease (George 2000). The clinical presentation of TTP and HUS are at times, very similar. Some patients who develop TTP due to the deficiency of ADAMTS13 can develop organ dysfunction, including a lack of renal function (Vesely, George et al. 2003). Alternatively, HUS can also occur in adult patients and involve neurological symptoms. The similarity in symptoms makes diagnosis difficult, where occasionally HUS can be misdiagnosed as TTP and *vice versa*.

1.1.5 Animal reservoirs for STEC

1.1.5.1 Cattle

Ruminants are considered to be the most important source of STEC human infections, due to their asymptomatically shedding of organism. Dairy cattle, as well as pastured and feedlot beef cattle, have been reported to shed STEC serotypes. Age plays an important factor where peak shedding is observed in post-weaning animals (Cray and Moon 1995). STEC O157 was isolated from less than 1.5% of calves under the age of 2 months, while in calves aged 4 months to 2 years, isolation ranged from 1.8% to 5% (Zhao, Doyle et al. 1995; Faith, Shere et al. 1996; Meyer-Broseta, Bastian et al. 2001). This demonstrates that STEC O157 is isolated 3 times more frequently from post-weaned animals than pre-weaned animals. After the age of two, shedding of STEC O157 appears

to decrease as cattle get older (Hancock, Besser et al. 1998). However, in Japan a higher level of shedding was seen in a study using polymerase chain reaction (PCR) to amplify *stx* genes of STEC serotypes from fecal samples of 204 calves and 306 adult cattle. A prevalence of 39% in calves under the age of 2 months, 79% in calves aged 2 to 8 months and 41% in cattle aged 1 year and older was reported (Shinagawa, Kanehira et al. 2000).

An increased prevalence of STEC is also seen during the summer and early autumn months (Bonardi, Maggi et al. 1999; Tutenel, Pierard et al. 2002). Summer temperatures provide a more suitable environment for the survival of STEC outside the host, which results in a source of infection and re-infection (Edrington, Callaway et al. 2006). A study described the collection of fecal samples monthly from 400 cattle, reported 4.8-36.8% shedding of STEC O157 where the highest level was found during late summer (Chapman, Siddons et al. 1997). Recently, a study was completed to determine if day length correlated with fecal shedding, as days are longer during summer months (Edrington, Callaway et al. 2006). After a period of 60 days, a significant difference in shedding was observed in the lighted pens compared to control groups with no light-treatment. Once the light-treatment was removed from the test group, shedding decreased to levels equivalent to the control group.

Various studies on the worldwide prevalence of STEC in cattle have been carried out. The majority of studies have focused on the shedding of STEC O157 because of its importance in human infections and outbreaks. The prevalence of STEC O157 ranged from 0% to as high as 85%, where concentrations varied from 4 to 10^7 colony forming units (CFU)/g, but 10 to 100 CFU/g is the average (Gyles 2007). In Japan, the duration and magnitude of serotype STEC O157 and O26 shedding was recently investigated in naturally infected cattle (Widiasih, Ido et al. 2004). The duration ranged between 10 weeks for O157 and 3 weeks for O26, where shedding varied from 10^4 CFU/mL for STEC O157 to 10^2 CFU/mL for STEC O26. Shiga toxin-producing *E. coli* O157 shedding in cattle has been described in many countries such as Argentina, USA, Brazil, Australia, Japan, Korea, China, and numerous European countries where diarrhea outbreaks are commonly described (Cobbold and Desmarchelier 2000; Fairbrother and Nadeau 2006). On the other hand, STEC O157 shedding was not reported in India or Thailand, where STEC is uncommon.

Non-O157 STEC serotypes are also regularly isolated from cattle. The prevalence of these organisms varies from 10% to 20%, but may reach as high as 80% to 90% (Fairbrother and Nadeau 2006). However, variability in detection is commonly due to the sampling techniques and sensitivity. The prevalence measurement of these strains is difficult since identification is based of the presence of *stx* genes. Numerous countries, including China, Uganda, Argentina, Scotland, Germany, Canada, and the USA, have reported shedding of non-O157 serotypes such as O26, O103, O111 and O145 (Kaddu-Mulindw, Aisu et al. 2001; Leung, Yam et al. 2001; Bettelheim 2003; Pearce, Evans et al. 2006). A recent survey in Scotland, which described the prevalence of non-O157 STEC serotypes in 6,086 fecal samples from 338 farms, identified shedding of STEC O26 in 23%, STEC O103 in 22% and STEC O145 in 10% of samples using immunomagnetic separation and slide agglutination (Pearce, Evans et al. 2006).

1.1.5.2 Other ruminant species

Ruminants other than cattle can play a significant role as STEC reservoirs. Sheep have been identified as an important reservoir, although prevalence of STEC is lower than in cattle. A study in the USA identified a transient STEC O157 prevalence of 31% in sheep (Kudva, Hatfield et al. 1996). Separate studies in European countries reported the prevalence of STEC O157 in sheep and lambs to range between 2.2% and 8.7% (Heuvelink, van den Biggelaar et al. 1998; Chapman 2000; Oporto, Esteban et al. 2008). Non-O157 serotypes are also important, as over 100 different STEC serotypes have been isolated from sheep (Urdahl, Beutin et al. 2003). Results from molecular amplification reported that 50% to 87% of sheep farms were positive for non-O157 STEC serotypes (Urdahl, Beutin et al. 2003; Oporto, Esteban et al. 2008). Shiga toxin-producing *E. coli* O157 has also been isolated from sheep meat and milk products (Chapman, Siddons et al. 1997).

A recent study demonstrated that goats can carry and spread STEC. A number of serotypes were isolated from 39% of individual goats tested, and found that all farms sampled tested positive for STEC (Vu-Khac and Cornick 2008). Shiga toxin-producing *E. coli* has also been frequently isolated from unpasteurized goat milk and cheese (Bielaszewska, Janda et al. 1997; Stephan, Schumacher et al. 2008). Buffalo are also

believed to be a important source of STEC infections in developing countries, where STEC O157 is commonly isolated from herds (Caprioli, Morabito et al. 2005). A study found that 27% of individual buffalo and 70% of farms sampled were positive (Vu-Khac and Cornick 2008). In Asian countries such as Vietnam, buffalo are raised by farmers for field ploughing, transportation of farm products and food production. Such practices increase the chances of human infection due to the close proximity between humans and animals.

Wild ruminants such as deer have also been suggested to play an important role as reservoirs for infection of domestic ruminants, since they frequently share the same pasture. Several groups have isolated STEC serotypes from deer populations. A USA study reported that 2.4% of white-tailed deer tested positive for STEC O157, while in Japan 1% of deer populations were positive (Asakura, Makino et al. 1998; Sargeant, Hafer et al. 1999). Several reports have also identified deer products such as meat and beef jerky as culprits in human infection (Keene, Sazie et al. 1997; Rabatsky-Ehr, Dingman et al. 2002). Although the prevalence of STEC in deer is low, their ability to roam pastures and farm land contributes to the spread of this organism, where wild deer were responsible for an outbreak from unpasteurized apple cider (Besser, Lett et al. 1993).

1.1.5.3 Other non-ruminant animals

Although the prevalence of STEC among non-ruminants is minimal, several species present a risk to humans. Pigs are described as an STEC reservoir, where the prevalence of STEC O157 associated with slaughtered pigs was found to range between 0% to 2% in USA and European countries (Johnsen, Wasteson et al. 2001; Bonardi, Brindani et al. 2003; Feder, Wallace et al. 2003; Oporto, Esteban et al. 2008). However, studies found an elevated STEC occurrence in Japan (14%) and South America (69%) (Rios, Prado et al. 1999; Kijima-Tanaka, Ishihara et al. 2005). These differences could be attributed to accidental exposure through contamination of manure and feed, or to poor husbandry and slaughter practices. Many of the isolated strains of STEC O157 are believed to be specific to pigs and associated with edema disease (Gannon, Gyles et al. 1988). It has also been suggested that rabbits may play a role as a STEC reservoir. At this

time only one outbreak has been reported as a result of rabbits (Pritchard, Williamson et al. 2001). Several studies investigated the prevalence of STEC in rabbits and found all samples to be negative (Rodriguez-Calleja, Garcia-Lopez et al. 2006; Assies, Eggenkamp et al. 2007).

House flies, which can carry and spread infectious organisms over vast areas, have been found to carry STEC (Sasaki, Kobayashi et al. 2000). These insects were believed to be responsible for an outbreak in a nursery school in a rural area of Japan in September 1996 (Kobayashi, Sasaki et al. 1999). Using electron microscopy, it was demonstrated that STEC O157 adhered to the surface of the house fly mouth and was able to proliferate. Shiga toxin-producing *E. coli* has also been recovered from several bird species. For example, strains were isolated from gull droppings collected from harbors in Japan and molecular analysis showed that the amino acid sequence of the avian Stx was closely associated with human-origin rather than animal-origin STEC (Makino, Kobori et al. 2000). Shiga toxin-producing *E. coli* strains which produce a variant of the Stx have also been regularly isolated from pigeons (Dell'Omo, Morabito et al. 1998). Neither birds nor house flies are thought to be true STEC reservoirs; instead they are considered potential vectors which can spread the organism by travelling through contaminated areas. Companion animals such as dogs, cats and even horses have been found to carry STEC O157 (Dell'Omo, Morabito et al. 1998). This could be a result of direct contact with farm animals or humans.

1.1.6 Modes of transmission

1.1.6.1 Environmental exposure

Environmental factors play a role in the exposure of animals and humans to STEC serotypes (summarized in Figure 1.2). Run-off water from dairy farms and pastures due to heavy rainfall can contaminate surface water such as ponds, rivers, lakes, springs and water supplying wells, commonly used for outdoor activities

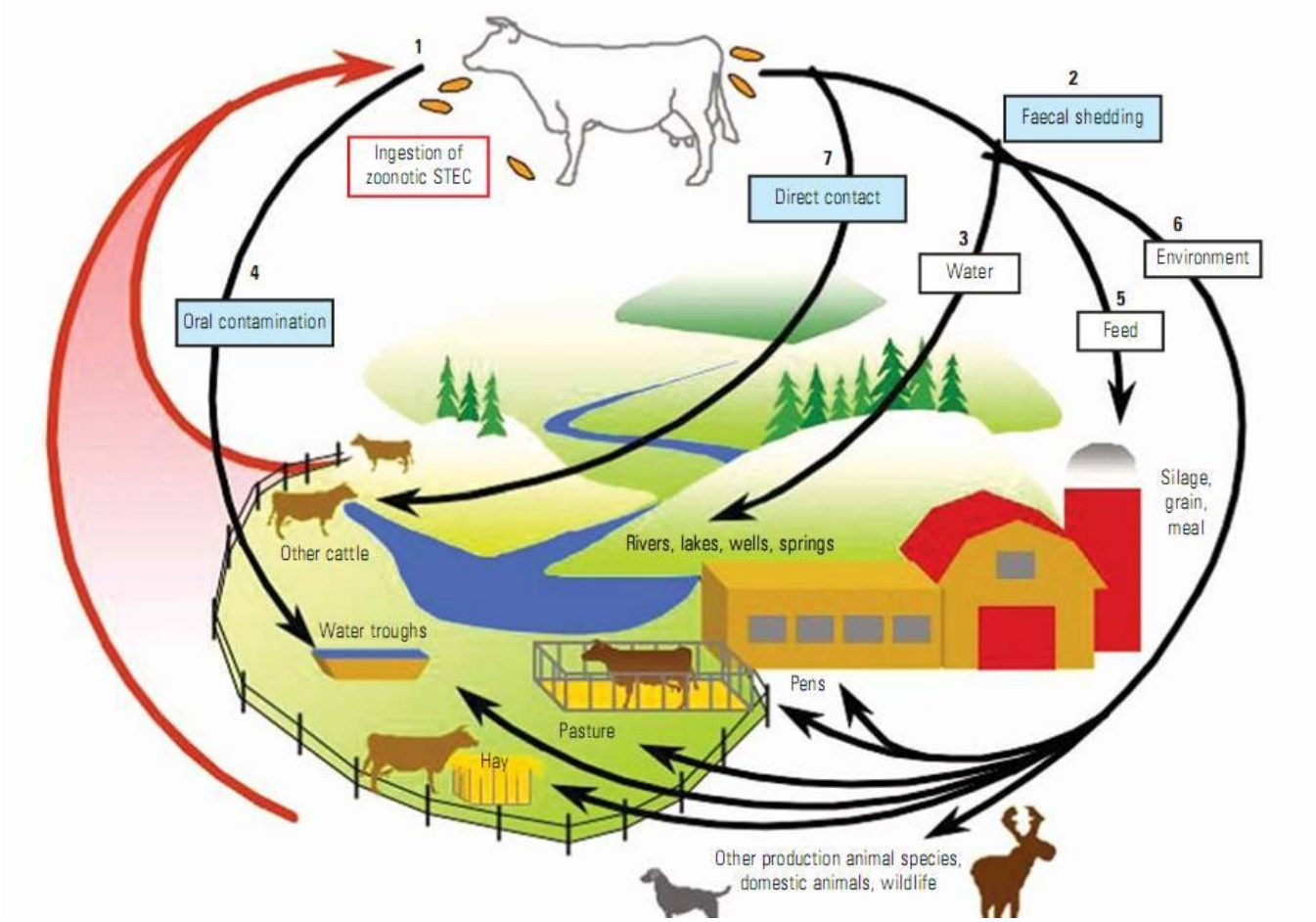


Figure 1.2 Sources of infection and spread of STEC in a farm environment. (1) Shiga toxin-producing *E. coli* is ingested by cattle or other ruminants. (2) The organism is then shed asymptomatically in fecal matter. (3) Run-off water from infected dairy farms and pastures, due to heavy rainfall can contaminate surface water such as ponds, rivers, lakes, springs and water supplying wells. (4) Water troughs can be easily contaminated by infected cattle, deer, birds and insects including flies. (5) Contamination of crop fields used for animal feed can occur through the spread of manure as fertilizer, fecal matter left by birds or wild ruminants, or from contaminated water run-off. (6) Fecal matter can also contaminate pastures, water troughs, animal pens and other animal species present in a farm environment, which in turn infect other cattle through their fecal remains. (7) Infection with STEC can also take place through direct contact between cattle. Diagram was adapted from (Fairbrother and Nadeau 2006).

(McGowan, Wickersham et al. 1989; McCarthy, Barrett et al. 2001; Neely, Bell et al. 2004). The dumping or spreading of sewage or wastewater from human origin can also play a role, since the persistence of STEC in soil, plants and plant roots allows the infection of cattle, and makes environmental exposure a hazard for human infections (Gagliardi and Karns 2002; Vernozy-Rozand, Montet et al. 2002). The practice of applying manure and slurry for fertilizer to crop land, and grazing is another source of contamination and re-introduction of STEC into cattle and other animals (Jiang, Morgan et al. 2002). The application of manure followed by heavy periods of rainfall, allow the bacteria to leach into lower layers of the soil, which can then drain into rivers or other bodies of water (Gagliardi and Karns 2000). Contamination of crop fields used for animal feed such as soybean meal, grass hay, silage grasses and grain pellets can also occur through the spread of manure as fertilizer, fecal matter left by birds or wild ruminants, or from contaminated water run-off (Fairbrother and Nadeau 2006). An STEC prevalence of 14.9% was found in animal feed collected and tested from 54 feedlots in the USA (Dodd, Sanderson et al. 2003). Feed can also become contaminated during transportation to the mill where a strain found at a mill was believed to have been cross-contaminated from a farm sampled more than 5 km away (Davis, Hancock et al. 2003). Silage, which is often fed to cattle and sheep, can play a role in the spread of STEC if the contaminated grass and poor silage management are combined (Fenlon and Wilson 2000).

Another important source of contamination is the water quality found in drinking troughs. This water can be easily contaminated through fecal matter or saliva from infected cattle, deer, birds and insects such as flies (Fairbrother and Nadeau 2006). Water troughs play a key factor in the spread of STEC, since the organism can survive and even thrive in fecal sediment found in troughs (LeJeune, Besser et al. 2001). Water contamination may also result from the original source, if water is not treated.

However, the main exposure to cattle, including pastures, pen floors, and water troughs, is the cattle themselves (Shere, Bartlett et al. 1998). Animal pens, where cattle are raised, play a significant role in the transmission of STEC. Cattle kept in pens were shown to shed STEC for a period of 4 months, while animals from the same farm which were kept on pasture, did not shed for the period tested (Jonsson, Aspan et al. 2001). This variation in shedding is a result of the exposure to the bacteria, where poor husbandry can

affect the persistence of STEC. Animals which are kept in feedlot pens, where the floor is muddy or urine and fecal matter is allowed to accumulate, demonstrated higher prevalence of shedding than animals raised in normal pens (Smith, Blackford et al. 2001). Interestingly, the way in which an alleyway is cleaned can also have an effect on shedding. Farm alleyways cleaned by water jets were 8 times more likely to test positive for STEC O157 than alleyways which were scraped (Garber, Wells et al. 1999).

1.1.6.2 Food and water

The most common method by which humans are infected with STEC is through the consumption of contaminated foods (Figure 1.3) (Rangel, Sparling et al. 2005). The most frequent sources are ground beef products such as uncooked hamburgers (Chinen, Tanaro et al. 2001). One of the largest STEC O157 outbreaks in North America occurred between December 1992 and January 1993, resulting in 732 cases throughout multiple states, where 195 patients were hospitalized and 4 died (Bell, Goldoft et al. 1994). This outbreak was traced to undercooked hamburgers served in a fast-food restaurant.

Other animal products such as cheese, butter, milk and other milk products have also been the cause of infection (Figure 1.3) (Gomez, Miliwebsky et al. 2002; Karns, Van Kessel et al. 2007). In addition, since the early 1990s, produce such as lettuce, spinach, salad, apple cider, sprouts, and coleslaw have become an important source of outbreaks (Itoh, Sugita-Konishi et al. 1998; Soderstrom, Lindberg et al. 2005). These products become contaminated by irrigation with contaminated water, or contact with manure or slurry of infected animals. Between 1995-2006, a total of 22 STEC O157 produce outbreaks were documented in the USA and the majority were traced back to lettuce and spinach (Cooley, Carychao et al. 2007). The most recent STEC outbreak was reported in September 2006 involving fresh baby spinach. This outbreak affected Canada and 26 USA states resulting in 205 cases and 3 deaths, and was attributed to cattle that tested positive for the same strain, a mile away from the spinach ranch (Jay, Cooley et al. 2007).

Outbreaks related to water sources tend to be of greater scale, as observed in 2000 in Walkerton, Ontario, when STEC O157 and *Campylobacter jejuni* contaminated the drinking water supply (Holme 2003). As a result, 2,100 individuals became ill and there

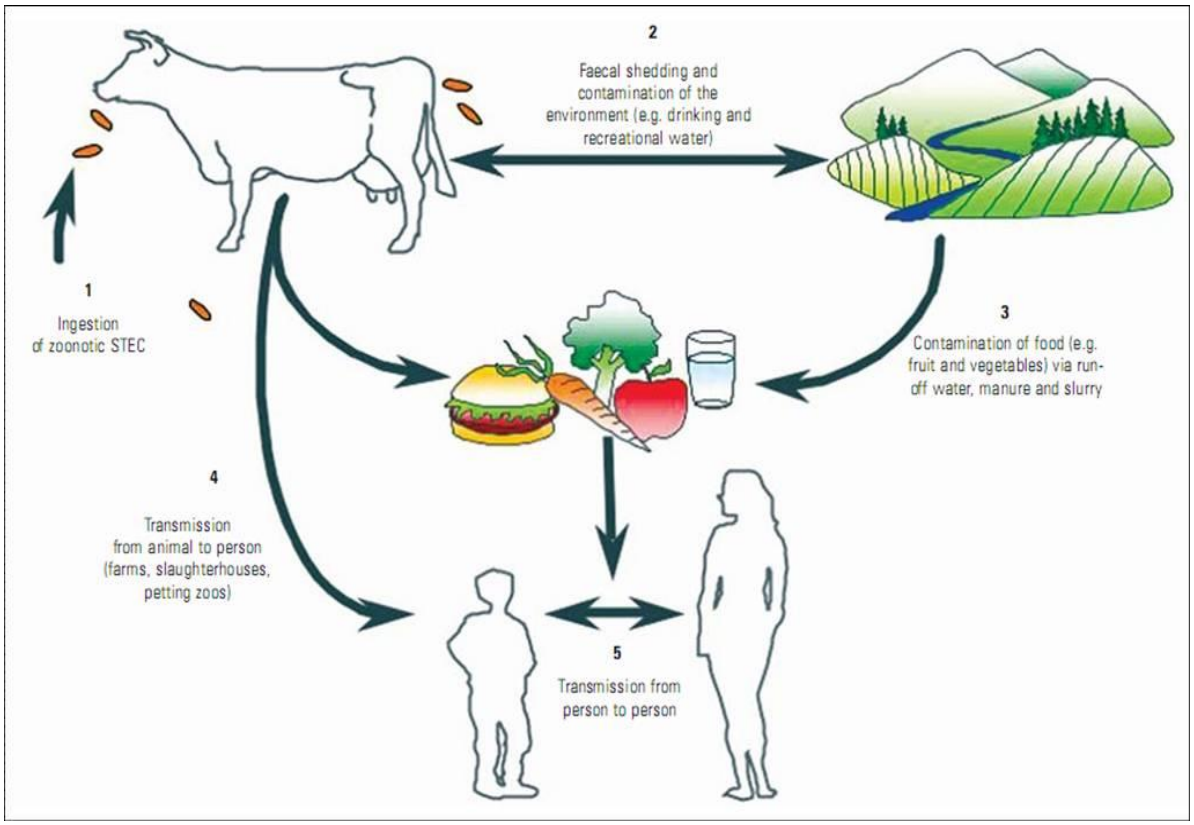


Figure 1.3 Human exposures to STEC. (1) Shiga toxin-producing *E. coli* is ingested by cattle or other ruminants. (2) The organism is then shed asymptomatically in fecal matter and contaminates surface water commonly used for drinking and summer outdoor activities such as swimming. (3) Vegetable products and fruit used for juice can become contaminated by irrigation with contaminated water or contact with slurry and manure of infected animals. Other animal products such as cheese, butter, and milk as well as ground beef contaminated at slaughter through infected carcasses, have also been a source of infection. (4) Individuals working at a farm and in slaughterhouses, petting zoos, and farm visits can lead to human infections through direct contact with animals or fecal matter. (5) Person to person distribution via the fecal-oral route is also an important source of transmission. Diagram was adapted from (Fairbrother and Nadeau 2006).

were 7 fatalities. Another large outbreak occurred in a small city in rural Missouri where the main water supply was not treated with chorine. This outbreak led to 243 cases where 32 patients were hospitalized resulting in 4 deaths (Swerdlow, Woodruff et al. 1992). Water supply outbreaks often occur after soiled surface water contaminates well water after heavy rainfall. These outbreaks tend to be of larger scale due to the accessibility of the contaminated water, and the incubation period of STEC O157 which can take up to 8 days.

1.1.6.3 Direct contact

Person-to-person spread via the fecal-oral route is an important source of transmission, especially in day care centres, schools, and swimming pools, individual homes and communities where children are in close proximity (Vogelsang and Pulz 1999). Poorly chlorinated water also plays a role in STEC infections where several outbreaks have been reported from swimming and paddling pools used for toddlers (Brewster, Brown et al. 1994; Hildebrand, Maguire et al. 1996; Friedman, Roels et al. 1999). Common practices such as "shocking" a pool, which involves dumping large quantities of chlorine into a pool, helps with algae control and keeping the water clear, but it does not fully inhibit bacterial growth (Friedman, Roels et al. 1999). Experimental work has shown that STEC O157 can survive in water for as long as 10 weeks in varying temperatures of 8ºC to 25 ºC (Wang and Doyle 1998).

Recently, petting zoos have been identified as an important source of transmission of STEC O157 to humans. Over the last few years the popularity of open farms and petting zoos has grown, where visitors including children can easily access various animals such as sheep, calves, rabbits, pigs, kittens and lambs. Practices such as feeding and handling these animals have lead to an increase in human infections. Between 2004 and 2005, three separate outbreaks of STEC occurred in an agricultural fair in North Carolina, a festival in Florida and a petting zoo in Arizona (DebRoy and Roberts 2006). A total of 173 cases were reported, including 22 cases of HUS. The outbreak in Florida was linked to goats, sheep and cows present at the petting zoo. Regular zoos have also begun to introduce interactive animal handling areas. These areas have led to human infections where two children were hospitalized following infection with STEC O157.

After officials sampled the area, they found that 15 of the 25 fecal samples collected contained STEC O157 (Stirling, Griffith et al. 2008). As a result, the interactive animal area was shut down.

Daycare centers are another source for STEC spreading. These centers are a prime location for direct contact between children from different locations. An outbreak in a Dublin Ireland daycare centre affected 10 out of 45 children and one staff member (O'Donnell, Thornton et al. 2002), while in Wales, a nursery reported 31 in 104 children became infected, resulting in 2 cases of HUS (Al-Jader, Salmon et al. 1999). The risk that these children will take the infection home or to school is quite high and can facilitate spread between children.

1.2 STEC virulence factors

Shiga toxin-producing *E. coli* contain a number of virulence factors that facilitate the colonization of a host. These factors include toxins, fimbrial adhesins, nonfimbrial adhesins, proteases and several elements found on the bacterial plasmids or chromosome (on Table 1.1).

1.2.1 Shiga Toxins

In 1977, it was reported that culture filtrates from several *E. coli* strains produced a cytotoxic effect on Vero cells that could be neutralised by antiserum to Stx from *S. dysenteriae* (Konowalchuk, Speirs et al. 1977; O'Brien, LaVeck et al. 1982). This observation led to the discovery that a pathogenic strain of *E. coli* O157:H7 produced Shiga-like toxins (O'Brien, Lively et al. 1983). Enteric pathogens such as *S. dysenteriae* and STEC, which harbour Stxs, play a significant role in disease and are responsible for extensive global morbidity and mortality (Kaper 1998; Paton and Paton 1998). For STEC, Stx is the main virulence factor involved in the development of HUS (Karmali, Steele et al. 1983; Karmali, Petric et al. 2004). The bloody diarrhea observed with STEC infection is attributed to the action of Stx on endothelial cells, thrombotic microangiopathy, and lesions formed on small blood vessels in the gut (Ray and Liu 2001).

Table 1.1 List of known virulence factors from Shiga toxin-producing *E. coli* (STEC). Table was adapted and modified from (Gyles 2007).

VIRULENCE FACTOR	PROPERTIES
Toxins	
Shiga toxins	Cytotoxic proteins / principal virulence factor of STEC / critical for the development of HUS
Haemolysin	Plasmid encoded RTX toxin / cytotoxic / lysing of host cells including hemoglobin / induces the release of inflammatory cytokines
Cytolethal distending toxin	Interferes with host cell cycle / heat-labile toxin / causes microvascular endothelial cell death
EAST1	Enteroaggregative heat-stable enterotoxin
Subtilase cytotoxin	Plasmid-encoded lethal cytotoxin
Fimbrial adhesins	
Lpf1	Long polar fimbriae / involved in microcolony formation and adherence / homology to the long polar fimbriae operon (lpf) of Salmonella enterica serovar Typhimurium
Lpf2	Thin fibrillae-like structures / involved in early stage adherence
Other fimbrial operons	Located in OI-1, OI-47, OI-141, OI-154 / involved in adherence
spf gene cluster	Pilus produced by sorbitol-fermenting STEC strains
HcpA	Type IV pilus / involved in adherence
YagZ	*E. coli* common pilus / found on all *E. coli* genomes sequenced to date/ involved in adherence
Nonfimbrial adhesins	
OmpA	Involved in adherence of both HeLa and Caco-2 cells
Efa1	Involved in adherence of cattle / involved in adherence, autoaggregation and RBC agglutination of hamster ovary cells
ToxB	Similar to toxins A & B in *C. difficile* / promotes the secretion of TTSPs
Saa	Auto-agglutinating adhesion found in LEE-negative STEC strains
Iha	Similar to iron-regulating IrgA protein found in V. *cholerae* / involved in adherence to epithelial cells
Secreted proteases	
EspP	Plasmid-encoded serine protease that cleaves pepsin A and human coagulation factor V / cytotoxic
StcE	Plasmid-encoded metalloprotease that cleaves C1-esterase inhibitor / involved in adherence / involved in the destruction of glycoproteins
NleA	Prophage-encoded serine protease that cleaves pepsin A and human apolipoprotein A-I / localises to the Golgi apparatus and compromises COPII-dependent protein trafficking and secretion
EpeA	Plasmid-encoded serine protease with mucinase activity / found in LEE-negative STEC strains
KatP	Plasmid-encoded catalase / peroxidase activity
Other	
etp gene cluster	Plasmid-encoded Type II secretion system
Urease	Found in *eae*-positive STEC strains / involved in acid tolerance
H21 flagellin	Involved in adherence
H6 flagellin	Involved in adherence
H7 flagellin	Involved in adherence / induction of inflammatory pathways
LPS	Induction of inflammatory cytokines

Shiga toxins belong to the family of AB_5 protein toxins. These toxins contain an enzymatically active A subunit, and 5 B subunits responsible for binding to the cell surface. Shiga toxins are made up of a single 32 kDa subunit A, which
is non-covalently bound to five 7.7 kDa B subunits which form a pentamer responsible for binding to a cellular receptor (O'Brien, Tesh et al. 1992). The A subunit consists of two minor subunits (A1 & A2) held together by a disulfide bond that is proteolytically cleaved by enzymes found in the endoplasmic reticulum (ER) and in the cytosol (Garred, van Deurs et al. 1995).

Shiga toxin-producing *E. coli* produces two Stxs originally identified as Stx1 and Stx2 (Strockbine, Marques et al. 1986). The genes encoding for the production of Stxs are found on toxin-converting lamboid prophages (O'Brien, Newland et al. 1984; Newland, Strockbine et al. 1985). The Stx1 protein is highly conserved and has a homology of over 98% to the Stx produced by *S. dysenteriae* type 1 (Takao, Tanabe et al. 1988; O'Loughlin and Robins-Browne 2001). The only apparent difference is a single amino acid located on the A subunit. A subtype of Stx1, called Stx1c, has been identified and is only isolated from *eae*-negative strains (Zhang, Bielaszewska et al. 2002). This subtype is found mainly in sheep STEC strains that cause mild or no disease (Brett, Ramachandran et al. 2003). The homology of Stx2 to Stx1 is less than 60%, due to the large variation in the amino acid sequence of the B subunit. In contrast to Stx1, several subtypes of Stx2 have been identified, consisting of Stx2c, Stx2d, $Stx2d_{act}$, Stx2e and Stx2f (Gyles 2007). Of 626 STEC isolates tested, Stx2d and Stx2e were located in *eae*-negative STEC strains, while subtype Stx2c was found in 5% of disease causing strains. Alternatively, none of the 626 STEC isolates contained Stx2f (Friedrich, Bielaszewska et al. 2002; Gyles 2007).

Shiga toxin receptors include globotriasoyl ceramide (Gb3), globotetraosyl ceramide (Gb4) and PI (a blood group glycolipid antigen) which is present in red blood cells (Jacewicz, Clausen et al. 1986; Lindberg, Brown et al. 1987; Lingwood, Law et al. 1987). The toxins bind to their glycolipid receptors in clathrin-coated pits and the Stx1–Gb3 complex is internalized by receptor-mediated endocytosis (Sandvig, Olsnes et al. 1989). This process involves the pinching of a fragment of the cell membrane, which produces a toxin-surrounding vesicle that fuses with lysosomal vesicles leading to the

destruction of the toxin. However, in cells which are susceptible to internalization, the toxin proceeds via the endosomal system and the trans-Golgi network, in a process that by-passes late endosomes (Mallard, Tang et al. 2002).

To inactivate ribosomes, the enzymatic A subunit of Stx must traverse the endoplasmic reticulum (ER) membrane. The A1 subunit is responsible for inhibiting protein synthesis in the cytosol by removing a specific adenine base from the 28S Ribonucleic acid (RNA) of the 60S ribosomal subunit (Sandvig 2001). This inhibition of protein synthesis can lead to apoptosis due to signalling by Stx or by the ribocytotoxic stress response (Cherla, Lee et al. 2003; Smith, Kane et al. 2003). In addition to its effect on eukaryotic cells, Stx has a similar effect on bacterial ribosomes resulting in decreased proliferation of susceptible bacteria such as *E. coli* (Suh, Hovde et al. 1998). This suggests that Stx may facilitate bacterial survival by inhibiting the growth of potential competitors in the lumen of the gastrointestinal tract.

Both the expression of Stx and the quantity of Gb3 receptor play an important role in disease. The increase of Gb3 receptor using sodium butyrate, which is found in the peripheral circulation and the colon, can increase the susceptibility of cells to Stx (Louise, Kaye et al. 1995). This treatment can also increase trafficking of the toxin to the nuclear envelope and the ER (Sandvig, Garred et al. 1992). Sodium butyrate increases the fatty acid chains of Gb3, which are important for intracellular translocation of Stx (Sandvig, Garred et al. 1996). Elements of the host inflammatory response to Stx, such as IL-1β and TNF-α can also increase in the synthesis of Gb3 (Louise, Kaye et al. 1995).

Although both Stxs share a significant homology, Stx2-producing strains are more likely to cause HUS than Stx1-producing strains (Kleanthous, Smith et al. 1990). One of the reasons for this is that Stx2 is 1000 times more toxic than Stx1 (Louise and Obrig 1995). In a mouse model, Stx2, when injected intravenously or intraperitoneally, required a LD_{50} that was approximately 400 times lower than that of Stx1 (Tesh, Burris et al. 1993). These observations were also confirmed when Stx2 was given intravenously to primates, were they developed progressive thrombocytopenia, hemolytic anemia and showed an increase in urinary IL-6 levels (Siegler, Obrig et al. 2003). On the other hand, animals injected with Stx1 showed no cytokine response, and no laboratory or clinical disease.

Once the crystal structures were established, four major differences were identified between the Stx2 and the Stx1 structure (Fraser, Fujinaga et al. 2004). First, a greater accessibility of the active site of the A subunit was found in Stx2 when compared to Stx1, which is blocked by part of the polypeptide chain of A2. Secondly, a difference in the carboxy terminus of the A1 peptide of Stx2 allows for binding at a receptor-binding site, while it remains unoccupied in the Stx1 structure. Thirdly, 1 of the 3 carbohydrate binding sites is different for Stx2 than for Stx1, and this conformational variability may lead to different binding affinities for the Gb3 receptor. Last, a variation between the two toxins is found in the carboxy terminus of the A subunit of Stx2, which forms a short 2-turn α-helix after passing through the pore of the B-pentamer, in contrast to the tail of the A subunit of Stx. This variability could result in different mechanisms of action that lead to the development of HUS.

1.2.2 Haemolysin

Haemolysin is a member of the pore-forming RTX family of toxins, produced by Gram-negative human or animal pathogens (Welch, Bauer et al. 1995). These toxins can be broad-range, lysing erythrocytes and nucleated cells from various species, or narrow-range, lysing only leukocytes in a species-specific manner. The gene encoding the hemolysin found in STEC O157 is present on a large 90 kbp plasmid, pO157, and consists of 4 open reading frames (ORFs) called the EHEC-*hlyCADB* operon, with a 60% homology to the *E. coli* α-hemolysin (Schmidt, Kernbach et al. 1996). A study reported that 92% of *eae*-positive strains harbored the hemolysin toxin while only 35% of *eae*-negative strains carried it (Eklund, Scheutz et al. 2001). It has been reported that all STEC O157 and 50% of STEC non-O157 strains tested where positive using PCR analysis for the *hlyB* gene (Schmidt, Beutin et al. 1995). This study also demonstrated that 19 out of 20 sera tested from HUS patients reacted with hemolysin, which confirms its production during the colonization of a host.

Hemolysin contributes to the pathogenesis of STEC by lysing hemoglobin which, in turn, releases iron. This iron is then taken up by STEC and used for bacterial growth. The α-hemolysin produced by *E. coli* is cytotoxic to leukocytes and fibroblasts, and it is thought that cytotoxicity and growth stimulation is its main role in *E. coli* virulence

(Cavalieri, Bohach et al. 1984). Initial characterization of the STEC hemolysin demonstrated its ability to lyse bovine and sheep erythrocytes as well as human RBCs (Bauer and Welch 1996). The STEC hemolysin is also understood to play a role in inflammation by inducing the production of interleukin-1β, which is believed to be one of the serum markers of HUS (Taneike, Zhang et al. 2002).

1.2.3 Other toxins

Shiga toxin-producing *E. coli* can express other enterotoxins which influence pathogenesis. Recently, a *cdt* gene cluster called *cdt-V*, responsible for a cytolethal distending toxin (CDT), was identified in STEC (Janka, Bielaszewska et al. 2003). The CDT belongs to a family of heat-labile toxins that interferes with the mammalian cell cycle, causes progressive cellular distension, arrest and eventual cell death (Cortes-Bratti, Karlsson et al. 2001). These genes are located on the chromosome with flanking regions homologous to lambda and P2 prophages, insinuating acquisition through phage transduction (Janka, Bielaszewska et al. 2003). The CDT gene cluster is made up of three genes called *cdtA*, *cdtB* and *cdtC*. The toxin is considered tripartite where the CdtB protein is the active A subunit of an AB_2 toxin, while the CdtA and CdtC make up the B subunit, which is required for the delivery of CdtB into the target endothelial cell (Lara-Tejero and Galan 2002).

The CDT is commonly found in sorbitol-fermenting O157 STEC strains. However, some non-fermenting STEC strains also possess this gene cluster. Of sorbitol-fermenting O157 STEC strains tested, 86.9% carried the cluster, while only 6% of non-sorbitol-fermenting O157 STEC strains harbored the gene cluster (Janka, Bielaszewska et al. 2003). STEC O157-purified CDT, when tested with human endothelial cells, resulted in irreversible damage. Using various endothelial cell lines, CDT toxin caused G_2/M cell cycle arrest, which led to inhibition of proliferation, cellular distension and cell death (Bielaszewska, Sinha et al. 2005). However, in a human microvascular endothelial cell line, this toxin caused caspase-dependent apoptosis resulting from blockage of G_2/M cycle. Interestingly, CDT is also capable of causing DNA damage via expression of the phosphorylated histone protein. Endothelial cells are found in lymphatic and blood

vessels and it has therefore been suggested that CDT plays a role in the systemic pathogenesis in combination with Stx or another toxin (Smith and Bayles 2006).

The enterotoxin EAST1 is also found in a large number of STEC strains. The EAST1 toxin initiates diarrhea by activating the cyclic GMP pathway that phosphorylates the chloride channels on the apical membranes of IECs (Uzzau and Fasano 2000). This phosphorylation stimulates chloride secretion, and the inhibition of sodium chloride absorption by the villus tip cells. This event leads to an increase in luminal ion content causing water to pull through the paracellular pathway, resulting in osmotic diarrhea (Nataro and Kaper 1998). Genetic analysis identified the *astA* gene encoding the EAST1 enterotoxin on all STEC O157 strains tested (Savarino, McVeigh et al. 1996). These results were also confirmed when two separate studies showed that 88% of 27 STEC (O111, O26 and O157) strains tested, and 100% of 67 (O157, O26, O111 and O145) strains, contained the *astA* gene (Paiva de Sousa and Dubreuil 2001). However, the sequence of EAST1 toxin in STEC strains differs from the hallmark sequence of the Enteroaggregative *E. coli* (EAEC) EAST1 toxin found on the EAEC strain O42, by possessing several mutations throughout the gene (Yamamoto, Wakisaka et al. 1997; Yamamoto and Taneike 2000).

A new subtilase cytotoxin has been recently identified in the STEC O113 serotype responsible for HUS outbreaks. This toxin is a member of a new family of AB toxins, where the A subunit has subtilase-like serine protease properties, and a homology to *Bacillus anthracis* (Paton, Srimanote et al. 2004). The injection of this toxin into mice caused extensive thrombosis, kidney, brain and liver damage resulting in death.

1.2.4 Fimbrial adhesins

The genome of STEC O157 possesses at least 16 fimbrial gene clusters (Low, Holden et al. 2006). However, under *in vitro* conditions, many of these genes are not functionally expressed. Of the small number of STEC clusters that are expressed, they produce multiple fimbrial adhesins believed to influence adherence and microcolony formation. Two adhesins closely related to the long polar fimbria operon (*lpf*) of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S*. Typhimurium), are *lpf1* and *lpf2* (Torres, Giron et al. 2002; Torres, Kanack et al. 2004). The *lpf1* operon

expresses long polar fimbriae, where an *E. coli* O157:H7 *lpf* mutant demonstrated a reduction in adherence and microcolony formation. The *lpf2* operon expresses thin fibrillae-like structures, and an isogenic mutant demonstrated a significant reduction in early stages of STEC adhesion. Both of these long polar fimbriae are located on the OI-141 and OI-154 chomosomal region of STEC O157, where at least four different variants of the *lpf* gene exist throughout STEC serotypes (Toma, Martinez Espinosa et al. 2004; Toma, Higa et al. 2006). It was recently described that fimbriae-encoding genomic islands such as OI-1, OI-47, OI-141 and OI-154 are distributed almost entirely in serotypes O157:H7 and O157:NM, which correlates with their ability to cause serious disease more frequently than other serotypes (Shen, Mascarenhas et al. 2005). Several other long polar fimbriae genes with homology to *sfaA* and *stcA* of *S.* Typhimurium have been identified, however further studies are required to fully understand their role in colonization (Spears, Roe et al. 2006).

Several pili have been identified in STEC serotypes, and are thought to play a role in the colonization of a host. A number of STEC O157 strains encode the pilus-forming *sfp* gene cluster found in sorbitol-fermenting strains (Friedrich, Nierhoff et al. 2004). This cluster is not found in non-sorbitol-fermenting STEC strains, or in *E. coli* O55:H7, an ancestor of STEC O157. Recently, a hemorrhagic *E. coli* pilus (type IV pilus) encoded by the chromosomal *hcpA* gene was discovered in STEC O157. This pilus bridges the bacterial cell to the host cells, and an *hcpA* mutant exhibited reduced adherence in human and bovine epithelial cells (Xicohtencatl-Cortes, Monteiro-Neto et al. 2007). Sera from HUS patients were also shown to react with the HcpA protein, confirming its expression during colonization. An additional pilus called the *E. coli* common pilus expressed from the *yagZ* gene, was identified in all *E. coli* genomes sequenced to date (Rendon, Saldana et al. 2007). This pilus is thought to bridge the bacterium and the epithelial cell, and a significant reduction in adherence was reported with a *yagZ* mutant.

1.2.5 Nonfimbrial adhesins

Shiga toxin-producing *E. coli* possess a number of nonfimbrial adhesins involved in pathogenesis. The outer membrane protein A (OmpA), which has been extensively studied in other *E. coli* strains and revealed to have adhesive properties, was shown to be

involved in the adherence of STEC O157 to HeLa and Caco-2 human cells (Torres and Kaper 2003). However, the host receptor to which this porin binds is unclear. The STEC protein called the factor for adherence (Efa1) is also important in adherence (Nicholls, Grant et al. 2000). An *efa1* STEC O111 mutant showed a reduction in cell autoaggregation, cell adherence and human RBC agglutination. These results were confirmed when STEC O111 and O5 *efa1* mutant were tested in calves, and demonstrated reduced shedding and a dramatic reduction in association with the intestinal epithelium (Stevens, van Diemen et al. 2002). Interestingly, these mutants also had reduced expression of the locus of enterocyte effacement (LEE) genes. A plasmid encoded gene called *toxB,* with similarities to toxins A and B found in *Clostridium difficile,* was found in a large number of STEC serotypes. The ToxB protein is believed to be involved in the adherence of STEC to epithelial cells by promoting the production of T3SPs (Tatsuno, Horie et al. 2001). In addition, with 47% homology to the *efa1* gene, *toxB* is believed to be a plasmid-encoded *efa1* homologue (Toma, Martinez Espinosa et al. 2004).

Several less-known adhesins are found throughout STEC strains. An auto-agglutinating adhesin called Saa (STEC autoagglutinating adhesin) was reported in LEE-negative strains responsible for HUS outbreaks (Paton, Srimanote et al. 2001). The transfer of *saa* on a plasmid to a non-pathogenic *E. coli* strain resulted in an increase in adherence nearly 10 fold that of the wildtype, while a STEC O103 *saa* mutant had a significant reduction in adherence. A study found that the *saa* gene was most commonly found in bovine and human STEC strains, suggesting a role in the colonization of cattle (Jenkins, Perry et al. 2003). The chromosomal *iha* gene found in a large population of STEC strains has also been reported to be involved in the adherence to epithelial cells (Tarr, Bilge et al. 2000; Toma, Martinez Espinosa et al. 2004). This gene is similar to the iron-regulated gene A (*irgA*) found in *Vibrio cholerae*.

1.2.6 Secreted proteases

A protease is an enzyme capable of cleaving a protein. Shiga toxin-producing *E. coli* possesses several plasmid-encoded proteases that facilitate the colonization of a host. An important serine protease found in STEC O157 and STEC O26 serotypes is the secreted EspP protein (Brunder, Schmidt et al. 1997). This protein is a member of the

autotransporter protein family, where homology is observed within *Neisseria*, *Haemophilus influenzae* and EPEC proteases. The EspP protein is capable of cleaving pepsin A and the human coagulation factor V, which contributes to mucosal hemorrhage. Another important protease is the metalloprotease StcE, found on the plasmid pO157 (Grys, Siegel et al. 2005). This protease cleaves the C1-esterase inhibitor that controls multiple inflammation pathways. Interestingly, the StcE protein is regulated by the LEE-encoded Ler protein and is believed to contribute to adherence and the destruction of glycoproteins.

Another protease which has been extensively studied is NleA (also known as EspI). This protein, which is prophage-encoded, has serine protease activity against pepsin A and human apolipoprotein A-I (Schmidt, Zhang et al. 2001). NleA is also regulated by the LEE-encoded Ler protein, and is present in 86% of LEE-positive STEC strains (Mundy, Jenkins et al. 2004; Roe, Tysall et al. 2007). Once NleA is secreted through the T3SS, it is localized to the Golgi apparatus, where it compromises COPII-dependent protein trafficking and secretion from the ER (Kim, Thanabalasuriar et al. 2007; Lee, Kelly et al. 2008).

Two lesser-known proteases have been discovered in STEC strains. A serine protease called EpeA, with both mucinase and protease activity, was found in the pO157 plasmid in the majority of LEE-negative STEC strains tested (Leyton, Sloan et al. 2003). Another protease called KatP is present on the pO157 plasmid, and is transported through the cytoplasmic membrane where it appears to have catalase-peroxidase activity (Brunder, Schmidt et al. 1996). Catalases are considered part of a bacterial defense mechanism against oxidative stress (Farr and Kogoma 1991).

1.2.7 Other

Shiga toxin-producing *E. coli* contains a number of other virulence elements which are important in host colonization. A gene cluster (*etp* genes) has been reported with homology to a Type II secretion pathway (Schmidt, Henkel et al. 1997). This cluster was identified in all STEC O157 serotypes, and in 60% of non-O157 serotypes tested. Several strains also carry the urease *ureC* gene found amongst *eae*-positive STEC isolates (Friedrich, Lukas et al. 2006). This gene is present amongst O111 and O145 serotypes,

but few strains actually express the gene. Urease expression by STEC serotypes may be involved in acid tolerance (Heimer, Welch et al. 2002).

The flagella produced by STEC can play an important role in pathogenesis. A study showed that H7 and H6 flagellin have adhesive properties that bind mucins I and II, and contribute to the colonization of cattle (Erdem, Avelino et al. 2007). These results were confirmed using a H7 *fliC* mutant with reduced capacity to adhere to a bovine terminal rectal epithelial cell line, but adherence was restored through complementation (Mahajan, Currie et al. 2008). However, complementation with the heterologous *fliC* from H6 was unable to restore H7 adherence properties. The ability of the wildtype to adhere was also reduced by the addition of H7-specific antibodies. The bacterial flagella can also play a role in the induction of inflammatory pathways, where the H7 from STEC O157 triggered intracellular signalling pathways and epithelial cell pro-inflammatory responses via TLR5 (Berin, Darfeuille-Michaud et al. 2002). This activation takes place by signalling the epithelial cell mitogen-activated protein (MAP) kinase and nuclear factor-kappa B (NF-κB) pathways, which results in Interleukin (IL)-8 secretion.

The lipopolysaccharide (LPS) produced by STEC, which is commonly released into the blood stream, plays a role in the establishment of HUS (Bitzan, Moebius et al. 1991; Azim, Qadri et al. 1996). Bacterial LPS is a strong activator of immune and non-immune cells, where the induction of signalling cascades, such as that via Toll-like receptor 4 (TLR4), result in the expression of chemokines such as IL-8 (Faure, Equils et al. 2000). Lipopolysaccharide and Stx, individually or in combination, cause THP-1 and human monocytes to release large amounts of IL-8 and other chemokines, such as IL-1β, tumor necrosis factor (TNF)-α, IL-8, GRO-β, macrophage inflammatory protein (MIP)-1α and MIP-1β, resulting in extensive platelet aggregation (Guessous, Marcinkiewicz et al. 2005). While exposure to Stx resulted in a 17 to 80 fold increase of chemokines, the addition of LPS combined with Stx, resulted in a 300 to 2000 fold increase. It is suggested that the development of HUS may in part be due to the combination of Stx and LPS, which causes the activation and release of chemokines by macrophages, resulting in tissue damage by leukocyte recruitment and activation.

1.3 Type III Secretion System

1.3.1 Overview

In 1994, Rosqvist and colleagues proposed a model for the secretion of Yop proteins by *Yersinia* into a host target cell (Rosqvist, Magnusson et al. 1994). This study led to the discovery of the Type III Secretion System (T3SS). This system is unique in function and structure in that it crosses not only the bacterial outer and inner membranes, but also the host epithelial cell membrane. The T3SS is a bacterial syringe-like structure, permitting the secretion of effector proteins into a host cell and allowing a pathogen to manipulate host cell pathways and functions.

A large number of Gram-negative pathogens possess T3SSs as a tool to colonize and infect a host. This system has been described in terms of colonization of a variety of hosts ranging from mammals to insects to plants (Troisfontaines and Cornelis 2005). The genes coding for the T3SS of many important enteric pathogens are located on either a plasmid or a pathogenicity island. The initial infection by *Salmonella* serovars is mediated by two T3SSs encoded by pathogenicity islands called *Salmonella* pathogenicity islands 1 and 2 (SPI-1) and (SPI-2). These systems mediate the secretion of effectors into the host cell cytoplasm which in turn manipulate host cell function and allow colonization, internalization and survival (Haraga, Ohlson et al. 2008). Other important enteric pathogens such as *Shigella flexneri*, uses a "trigger mechanism of entry" involving the formation of a macropinocytic pocket similar to *S. typhimurium*, where a plasmid-encoded T3SS secretes a number of effectors into IECs, which mediates invasion (Jensen, Harty et al. 1998). Although *Yersinia* spp, *S. typhimurium* and *S. flexneri* invade Microfold cells (M cells), *Y. enterocolitica* differs in the mechanism used to rearrange the actin cytoskeleton for bacterial uptake. While *S. typhimurium* and *S. flexneri* both use a T3SS to secrete effectors into host cells causing ruffling and engulfment (Grassl, Bohn et al. 2003), entry into M cells by *Y. enterocolitica* is dependent on adhesins to adhere to the host cell (Bliska, Copass et al. 1993; Clark, Hirst et al. 1998). This pathogen then uses its plasmid-encoded T3SS to mediate antiphagocytosis by secreting effectors into phagocytic cells (Cornelis, Boland et al. 1998).

1.3.2 Locus of Enterocyte Effacement

1.3.2.1 Shiga toxin-producing *E. coli*

The ability of STEC serotypes to effectively colonize intestinal eukaryotic cells and form the hallmark attaching and effacing (A/E) lesions, is a result of a chromosomal genetic element called LEE. An A/E lesion is characterized by the localized destruction of the brush border microvilli, and the intimate attachment of the bacterium to the host cell apical membrane, resulting in the formation of a pedestal-like structure underneath the bacteria (Frankel, Phillips et al. 1998). This 43 kb pathogenicity island, contains the genes involved in the formation of a T3SS (McDaniel, Jarvis et al. 1995; Perna, Mayhew et al. 1998). The STEC O157 LEE Island is integrated on one end with a 7.5 kb cryptic P4 prophage, which together, is inserted within the *selC* tRNA locus (Wieler, McDaniel et al. 1997; Perna, Mayhew et al. 1998). The LEE island contains 41 ORFs organized in five polycistronic operons called LEE1 through LEE5 (Wieler, McDaniel et al. 1997). The operons LEE1, LEE2 and LEE3 contain 22 ORFs that encode proteins required for the basal Type III Secretion apparatus, spanning the inner and outer membranes (Elliott, Wainwright et al. 1998; Perna, Mayhew et al. 1998; Roe, Hoey et al. 2003). These operons also include EscN, the ATPase of the secretion system, and the outer membrane porin EscC. The LEE 4 operon encodes the needle *escF* gene, the Esp (*E. coli* secreted proteins) group of genes, including the monomer *espA* filament, *espB, espD* and *espF* (Ebel, Podzadel et al. 1998; Kresse, Rohde et al. 1999; Wachter, Beinke et al. 1999; Ide, Laarmann et al. 2001; Shaw, Daniell et al. 2001; Wilson, Shaw et al. 2001; Roe, Hoey et al. 2003). These genes (excluding *espF*) permit the configuration of a needle-like structure, known as a translocon, and a pore to be formed, which allows effector proteins to be secreted directly into the host cell. The last operon contains the ligand intimin, the translocated intimin receptor (Tir), and its chaperone CesT (Kenny, DeVinney et al. 1997; Dean-Nystrom, Bosworth et al. 1998; Abe, de Grado et al. 1999; Elliott, Hutcheson et al. 1999; Cornick, Booher et al. 2002; Roe, Hoey et al. 2003).

However, the LEE pathogenicity island has been found to be inserted in other locations, aside from the tRNA locus, for a number of non-O157 serotypes. The STEC O26 bovine strain 413/89-1 is located in the *pheU* tRNA gene, while the STEC O103

bovine strain RW1374 had the LEE Island inserted in the *pheV* tRNA gene (Jores, Rumer et al. 2004). While the core region of these non-O157 LEE islands is conserved, the flanking regions differ where a number of unknown genes are present.

1.3.2.2 Enteropathogenic *Escherichia coli* and *Citrobacter rodentium*

Enteric pathogens other than STEC also contain the LEE pathogenicity island. In fact, the LEE Island was originally identified and sequenced in enteropathogenic *Escherichia coli* (EPEC) (Elliott, Wainwright et al. 1998). This pathogen is the leading cause of watery diarrhea in infants in developing countries, where mortality ranges from 10% to 40% (Levine 1987; Nataro and Kaper 1998; Chen and Frankel 2005). Unlike STEC, EPEC does not cause bloody diarrhea or HUS due to a lack of Stxs. However, while STEC is mostly asymptomatic in animals, EPEC is responsible for ruminant disease because of numerous features such as virulence factors such as bundle-forming pili (Fischer, Maddox et al. 1994; Cid, Ruiz-Santa-Quiteria et al. 2001; Jores, Rumer et al. 2004).

The EPEC LEE Island is smaller than that found in STEC, with a size of 35.5 kb, as it does not contain the 7.5 kb cryptic P4 prophage found on one end of the STEC LEE Island (Perna, Mayhew et al. 1998; Jores, Rumer et al. 2004). However, the LEE Island is still inserted within the *selC* tRNA locus, and contains all 41 ORFs organized into operons LEE1-LEE5. A high level of sequence homology within the 41 ORFs of STEC and EPEC is observed, where the majority average over 95% homology (Perna, Mayhew et al. 1998). However, a number of genes (*eae*, *espA*, *espB*, *espD* and *tir*) which express proteins that are exposed on the outer surface of the bacterium have a sequence difference of over 15%. The variability among these genes could be a result of the interaction with the host, causing an increase in selective pressure (Frankel, Phillips et al. 1998).

Another bacterial pathogen which contains the LEE island within its chromosome is *Citrobacter rodentium*. Unlike STEC and EPEC, this pathogen infects rodents and causes transmissible murine colonic hyperplasia in mouse breeding colonies (Schauer and Falkow 1993). The *C. rodentium* LEE Island is flanked by a ABC transport system operon and an IS element on one side, and a sequence homologous to the pO157 plasmid on the other side (Deng, Li et al. 2001). The presence of plasmid sequences flanking the

LEE island insinuates that horizontal plasmid transfer might be responsible for the A/E phenotype in *C. rodentium*.

The *C. rodentium* LEE Island contains all 41 ORFs, but differs in the organization of the *rorf1* and *espG* genes (Deng, Li et al. 2001). However, a conserved role in pathogenesis and disease has been described, where the intimin protein from EPEC restored virulence of a *C. rodentium intimin* mutant, while the Tir protein from STEC O157 restored virulence of a *C. rodentium tir* mutant (Deng et al. unpublished data) (Frankel, Phillips et al. 1996). Since this organism produces A/E lesions when colonizing the gastrointestinal tract, it has provided an excellent *in vivo* model to investigate the interaction between pathogen and host. In fact, many of the early studies exploring the role of Intimin and the LEE islands in virulence as well as the present studies on the role of LEE and non-LEE effectors have been investigated using the *C. rodentium* mouse model (Schauer and Falkow 1993).

1.3.3 Other pathogenicity islands

Apart from the LEE pathogenicity island, STEC serotypes possess other pathogenicity islands associated with virulence and disease. The sequencing of the STEC O157 genome confirmed the existence of multiple O islands (OI) such as OI-1, OI-43, OI-48, OI-115, OI-122, OI-140, OI-141 and OI-154 (Perna, Plunkett et al. 2001). Pathogenicity Islands such as LEE, and their role in virulence have been well-characterized. However, the elucidation of the role of these OIs in the virulence of STEC is still in its early stages. One exception is the well-characterized OI-122.

The OI-122 is a 23 kb pathogenicity island composed of 26 ORFs, harboring a number of virulence genes (Perna, Plunkett et al. 2001). These genes are divided into three modules, the first includes a gene with homology to the *S. Typhimurium pagC* gene involved in survival within macrophage, the second module contains the *nleB* and *nleE* secreted effectors and the *sen* gene resembling a *Shigella* enterotoxin, while the third contains the *efa1* and *efa2* genes used for adherence (Nicholls, Grant et al. 2000; Karmali, Mascarenhas et al. 2003; Nishio, Okada et al. 2005; Konczy, Ziebell et al. 2008). Interestingly, not all of these genes are found in every STEC serotype. Each module is believed to have been acquired or lost as a tranposon-like independent mobile

element. This is confirmed by the variable regions on the genome, where OI-122 is incorporated. For example, in STEC O157, OI-122 is adjacent to the *pheV* tRNA locus and is located 7 kb upstream of LEE island, while in STEC O26:NM, which contains module 2 and 3, the OI-122 is attached to LEE, resulting in a large 59.4 kbp pathogenicity island called LEE O26 (Karmali, Mascarenhas et al. 2003; Muniesa, Schembri et al. 2006). In STEC O103 serotypes, the OI-122 with module 2 and 3 is 43 kb upstream of LEE forming a large 111 kb island, while in STEC O113:H21, the one module containing OI-122 is entrenched in OI-48 (Shen, Mascarenhas et al. 2004; Jores, Wagner et al. 2005). The LEE and the OI-122 islands are functionally related where *nleB* and *nleE* genes transcribe effectors that are secreted through the T3SS found on the LEE Island (Kelly, Hart et al. 2006).

In 2003, Karmali and colleagues devised a method to classify STEC serotypes into five seropathotypes A through E, based on the distribution of the OI-122 Pathogenicity island modules (summarized in Table 1.2) (Karmali, Mascarenhas et al. 2003; Wickham, Lupp et al. 2006). The OI-122 was selected given that its presence in STEC correlates with a strain's ability to cause HUS and trigger outbreaks. These seropathotypes were divided as follows (Karmali, Mascarenhas et al. 2003): Seropathotype A consisting of STEC O157:H7 and O157:NM is affiliated with human disease involving outbreaks and HUS cases. Seropathotype B consisting of serotypes O26:H11, O103:H2, O111:NM, 121:H19 and O145:NM are associated with outbreaks of HUS but are less common than serotype O157:H7. Seropathotype C consists of serotype O91:H21, O104:H21 and O113:H21, and are associated with sporadic cases of HUS, but not involving outbreaks. Seropathotype D involves serotypes associated with diarrhea but not with HUS cases, while seropathotype E consist of serotypes not implicated in human disease.

A number of genes (*pagC, nleB, nleE, ent* and *efa1*) located on the OI-122 Island play a critical role in the ability of a pathogen to colonize, and cause outbreaks involving HUS (Wickham, Lupp et al. 2006; Wickham, Lupp et al. 2007). A *C. rodentium nleB* mutant required an infectious dose 285-fold higher than the wildtype strain (Wickham, Lupp et al. 2006). Since the *nleB* gene is found in 100% of STEC O157:H7 and 69% of non-O157 strains implicated in outbreaks with HUS, and that a low infectious dose plays

Table 1.2 Classification of STEC serotypes into seropathotypes. * Represents HUS and hemorrhagic colitis. Diagram adapted and modified from (Karmali, Mascarenhas et al. 2003)

Seropathotype	Relative incidence	Frequency of involvement in outbreaks	Association with sever disease *	STEC Serotypes
A	High	Common	Yes	O157:H7, O157:NM
B	Moderate	Uncommon	Yes	O26:H11, O103:H2, O111:NM, 121:H19, O145:NM
C	Low	Rare	Yes	O91:H21, O104:H21, O113:H21, others
D	Low	Rare	No	Multiple
E	non-human only	Not applicable	Not applicable	Multiple

a significant part in outbreaks; this protein could be a vital to STEC pathogenesis. In fact, genes such as *nleB* and *efa1* are used in a clinical setting for molecular serotype typing to identify pathogenic serotypes, apart from Mackonkey sorbitol plating and other commonly used STEC identification methods.

Although less-characterized, other pathogenicity islands found within the STEC genome also contain important virulence factors. The *lpf1* operon and the *lpf2* operon which express long polar fimbriae and thin fibrillae-like structures, respectively, are involved in adherence and microcolony formation. Both of these long polar fimbriae are located on the OI-141 and OI-154 chromosomal region of STEC O157, where at least four variants of *lpf* exist throughout STEC strains (Toma, Martinez Espinosa et al. 2004; Toma, Higa et al. 2006). As noted, fimbriae-encoding genomic islands such as OI-1, OI-47, OI-141 and OI-154 are distributed almost entirely in serotypes O157:H7 and O157:NM, indicative of their ability to cause serious disease more frequently than other serotypes (Shen, Mascarenhas et al. 2005).

The Iha is an adherence-conferring protein produced by STEC O157 (Tarr, Bilge et al. 2000). This protein, which is not fully understood, is adjacent to the tellurite resistance loci, has a conserved structure, and has duplicate genes located on OI-43 and OI-48 (Tarr, Bilge et al. 2000; Toma, Martinez Espinosa et al. 2004). Both of these islands are identical and contain over 100 ORFs. The uncharacterized Z1640 gene located in the middle portion of the OI-48 has also recently been tested and suggested to play a role in virulence (Shen, Mascarenhas et al. 2004). Using PCR, 36 STEC strains were sampled, and the intact gene Z1640 was only located in serotypes such as O157:H7, O26:H11, O103:H2, O111:NM, and O145:NM, commonly associated with HUS and worldwide outbreaks. On the other hand, in STEC strains that do not cause human infection, such as O91:H21 and O113:H21, the Z1640 gene was found only in fragmented forms.

1.3.4 Structural components and assembly of secretion apparatus

The Type III Secretion apparatus is made up of two interconnected rings, spanning both the inner and the outer membranes. A translocon is then extended from the

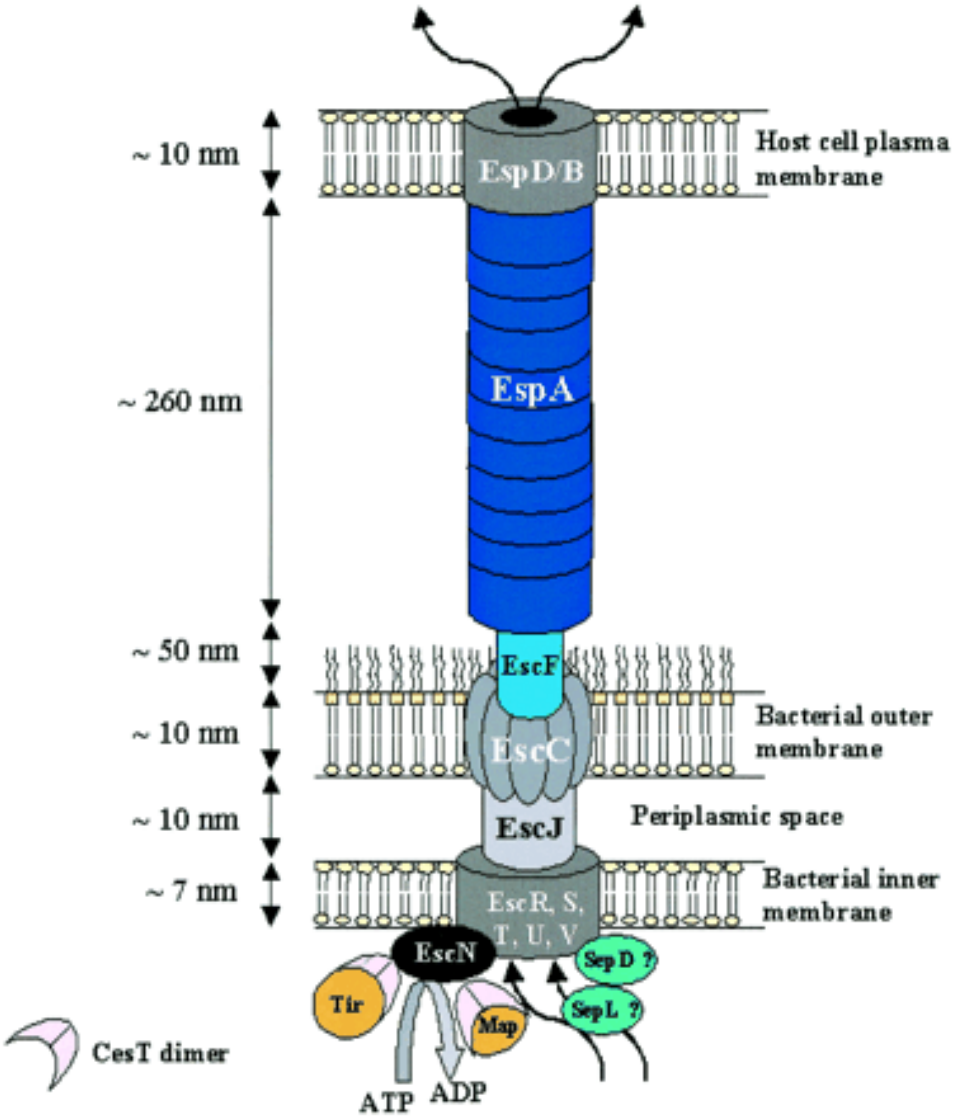


Figure 1.4 Representation of the Type III Secretion Sytem (T3SS). The EscC protein is the main component of the outer membrane ring. This ring uses the EscJ protein to form a periplasmic ring that connects the outer membrane ring to the inner membrane. The inner membrane rings, are made up of a group of proteins called EscV, EscR, EscS, EscU and EscT. The EscF protein forms the needle structure connecting the EscC outer membrane rings to the extracellular space. A filament then extends from the needle made up EspA protein subunits. Two other proteins called EspD and EspB form a pore on the surface of the host cell, and connect the filament to the eukaryotic cell. The EscN protein is an ATPase which provides the energy for the transportation of effectors through the channel. SepL and SepD are cytoplasmic components of the T3SS. Diagram was adapted from (Garmendia, Frankel et al. 2005).

bacterial outer membrane, allowing the secretion of proteins into a host cell (summarized in Figure 1.4) (Roe, Hoey et al. 2003). The initial formation of the membrane structure, involves proteins with a sec-dependent signal sequence, which allows them to be transported and positioned on the membranes through the sec- pathway. Several cytosolic proteins are then added to the basal end of the structure. The next portion of the apparatus assembles in a sec-independent method, where proteins are exported through the channel formed by the interconnecting membrane structure.

The EscC protein is the main component used for the outer membrane rings (Ogino, Ohno et al. 2006). This protein belongs to the secretin super family of outer membrane proteins, which uses multiple subunits to form a two ring-shaped homomultimeric complex on the outer membrane (Koster, Bitter et al. 1997; Garmendia, Frankel et al. 2005). The inner membrane rings are made up of a group of proteins called EscV, EscR, EscS, EscU and EscT (Garmendia, Frankel et al. 2005). At this point, little is known about their interactions and their roles with respect to one another. However, the EscV protein, which possesses seven transmembrane domains, is the principal protein in the formation of the inner membrane rings. This was confirmed when an *escV* mutant lost the ability to form the apparatus, causing the accumulation of the EscC protein in the periplasm (Gauthier, Puente et al. 2003; Garmendia, Frankel et al. 2005).

The STEC EscJ protein makes up the large 24-subunit periplasmic ring that connects the outer with the inner membrane structure (Yip, Kimbrough et al. 2005). This protein is required for the transportation of numerous proteins, where an *escJ* mutant failed to secrete or form a functional T3SS (Garmendia, Frankel et al. 2005). Using nuclear magnetic resonance spectroscopy, the EscJ protein was found to possess two domains connected by a linker, which fit perfectly into the width of the periplasmic region of Gram negative bacteria (Crepin, Prasannan et al. 2005). The EscJ protein spans both membranes, but appears to mostly interact with the outer membrane structure and with the EscF needle protein (Ogino, Ohno et al. 2006). This interaction may be required to directly connect the channel to the needle structure. Interestingly, a homologue of EscJ has been discovered in *Salmonella* spp, called PrgK that with the assistance of another protein, is required for the formation of the inner membrane rings, and may be involved in bringing together the periplasmic channel protein with the inner membrane rings

(Kimbrough and Miller 2000; Yip, Kimbrough et al. 2005). All three of the structural membrane proteins discussed (EscC, EscV and EscJ), have *sec-dependent* sequence signals, and have structural homologues found in other T3SSs possessing bacteria such as *Salmonella*, *Shigella* and *Yersinia* spp. (Schuch and Maurelli 1999; Galan 2001; Ghosh 2004; Kostakioti, Newman et al. 2005; Spreter, Yip et al. 2009).

For proteins to be secreted through the recently formed membrane apparatus, an energy source is required. The EscN protein is a unique ATPase which provides the necessary energy for the transportation of effectors (Gauthier and Finlay 2003). This protein also acts as an inner-membrane gate operator by interacting with chaperone-effector complexes through a docking site on its C-terminal domain. Through ATP hydrolysis, the conformational change of the domain may disrupt the chaperone binding site, allowing the effector to dissociate and be secreted through the T3SS (Woestyn, Allaoui et al. 1994; Akeda and Galan 2004; Zarivach, Vuckovic et al. 2007). This ATPase shares homology with the β subunit of the F0F1ATPase complex, which breaks down ATP in the membranes of both prokaryotes and eukaryotes (Zarivach, Vuckovic et al. 2007) . The EscN protein has also been found to interact with several proteins on the T3SS apparatus.

The single protein needle structure which connects the EscC outer membrane rings to the extracellular space is made up of an EscF protein (Wilson, Shaw et al. 2001). An *escF* mutant completely abolished the secretion by the T3SS, which was fully restored once the *escF* gene was re-introduced (Wilson, Shaw et al. 2001). These results demonstrated that without the EscF protein, the channel within the inner and outer membrane is inactivated or blocked. This protein shares a homology with the PrgI needle produced by *Salmonella*, where both needles protrude roughly 40 to 50 nm from the bacterial surface (Daniell, Takahashi et al. 2001; Marlovits, Kubori et al. 2004). In addition, like *Salmonella* and *Shigella*, the needle length is believed to be strictly regulated (Marlovits, Kubori et al. 2004; Kenjale, Wilson et al. 2005; Marlovits, Kubori et al. 2006). However, the regulatory protein which controls the STEC EscF length has not yet been discovered.

A trait which separates the STEC T3SS from the rest of the Gram negative T3SSs, is the production of an extended filament that binds directly to the EscF needle

structure (Knutton, Rosenshine et al. 1998; Delahay, Knutton et al. 1999; Wilson, Shaw et al. 2001; Daniell, Kocsis et al. 2003). This filament is made up of a polymer of multiple coiled-coil interactions between EspA protein subunits (Delahay, Knutton et al. 1999). The EspA subunits are added to the growing tip of the filament as they are secreted through the membrane-spanning channel (Crepin, Shaw et al. 2005). These subunits co-polymerize to form a hollow tube, which allows effector proteins to be secreted. This function was recently verified where the translocated intimin receptor (Tir) was secreted through the filament tip into the extracellular environment (Crepin, Shaw et al. 2005). Through close analysis of the crystal structure, the hollow tube is made up of roughly 28 subunits, with an inner diameter of 25 angstrom, an outer diameter of 120 angstrom, where each subunit gives a rise of 4.6 angstrom (Daniell, Kocsis et al. 2003). Just like the EscF protein, the extension of the EspA filament is also believed to be tightly regulated by controlling the amount of monomeric EspA available in the cytoplasm. This was confirmed where an increase in the concentration of intracellular EspA subunits resulted in an extended filament structure (Crepin, Shaw et al. 2005).

Once the translocon structure is complete, the filament, with the assistance of the EspB and EspD proteins, is inserted into IECs, where LEE and non-LEE effectors are secreted much like fluid through a syringe (Knutton, Rosenshine et al. 1998; Daniell, Delahay et al. 2001). Once effector genes are expressed and begin to be transported into a host cell, the transcription of *espA* is down-regulated (Dahan, Knutton et al. 2004). The EspB and EspD proteins are important in the secretion process because of their ability to form a pore on the surface of IECs (Fivaz and van der Goot 1999; Warawa, Finlay et al. 1999). Both proteins have been purified from eukaryotic cells, and demonstrate hemolytic properties on RBCs (Wachter, Beinke et al. 1999; Daniell, Delahay et al. 2001; Shaw, Daniell et al. 2001). The observed hemolysis was dependent on a working secretion system and the EspD protein. However, an *espB* mutant was still able to cause hemolysis, but levels were significantly reduced. These results show that EspD is essential for the production of the pore, and that EspB binding to EspD allows the pore to be fully formed. Interestingly, an *espD* mutant secreted low levels of the EspA filament and displayed reduced attachment to IECs when compared to the wildtype (Knutton, Rosenshine et al. 1998; Daniell, Delahay et al. 2001). It is believed that EspD plays a role

in attaching the filament to the IEC membrane, and could be involved in the capping of the EspA filament (Delahay, Knutton et al. 1999; Fivaz and van der Goot 1999).

1.3.5 Regulation of secretion by the Type III Secretion System

Pathogenic bacteria must be able to respond accordingly to any type of environment. This responsiveness becomes critical for the expression of virulence factors once the bacteria have reached a potential host. The regulation of the formation of A/E lesions is a complex system involving numerous direct and indirect regulatory factors (Figure 1.5). The T3SS found on the LEE Island is responsible for the secretion of proteins into host epithelial cells, which initiates the formation of A/E lesions. These lesions can be mimicked *in vitro* during early exponential growth phase by stimulating the bacteria to 37ºC, which allows lesion formation on HeLa cells (Rosenshine, Ruschkowski et al. 1996). However, this event does not occur if the bacteria are initially incubated at 27ºC.

Under non-host conditions such as the external environment, or during the initial time of ingestion, the expression of the LEE Island is suppressed by H-NS (Histone-like nucleoid structuring protein) (Umanski, Rosenshine et al. 2002). The H-NS is a negative regulator that responds to numerous regulatory proteins and environmental signals (Atlung and Ingmer 1997). This protein binds and represses Ler (LEE-encoded regulator), and the expression of LEE1, LEE2, LEE3, LEE4 and LEE5 operons at 27ºC (outside environment). This effect involves the binding of silencer regulatory sequences 1 and 2 (SRS1 & SRS2) that flank the LEE operon promoters (Bustamante, Santana et al. 2001). This binding results in the formation of a repressor nucleoprotein complex, which is stabilized by the H-NS-H-NS bridging interaction (Bustamante, Santana et al. 2001). Once the temperature is switched from 27ºC to 37ºC (gastrointestinal environment), Ler activates the LEE operons through the release of the H-NS mediated repression, by acting as a anti-H-NS factor (Umanski, Rosenshine et al. 2002). The Ler protein binds to the same H-NS region on LEE2 and LEE5, resulting in silencing through disruption of binding (Haack, Robinson et al. 2003; Torres, Lopez-Sanchez et al. 2007). The binding of Ler to the SRS1 weaken the H-NS nucleoprotein complex which allows the expression of

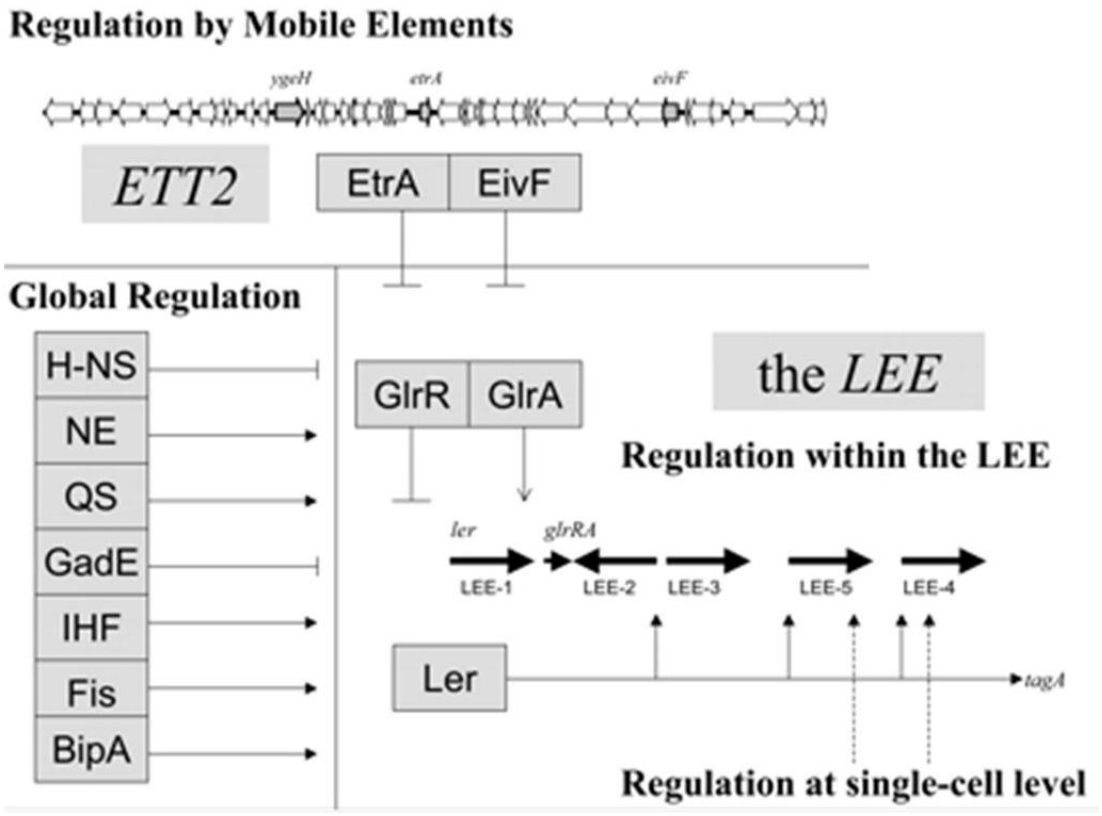


Figure 1.5 Transcriptional regulation of the locus of enterocyte effacement (LEE). Solid arrows represent activators, while crossbars represent repressors. For further explanation of LEE repressors or activators please see text. H-NS (Histone-like nucleoid structuring protein), Ler (LEE-encoded regulator), IHF (integration host factor), QS (quorum sensing), NE (norepinephrine). Diagram adapted and modified from (Zhang, Chaudhuri et al. 2004).

the LEE operons (Bustamante, Santana et al. 2001; Barba, Bustamante et al. 2005; Torres, Lopez-Sanchez et al. 2007).

The Ler protein, which is encoded on the LEE1 operon, is the central regulator of the LEE Island. A study has shown that the deletion of the *ler* gene limits production of the hallmark A/E lesions, and strongly attenuates virulence, making it a global regulator (Elliott, Sperandio et al. 2000). Using a HEp-2 cell *in vitro* model, a study has shown that the transcription of *ler* only appears to be required during the early stages of infection (Leverton and Kaper 2005). The transcription of LEE3, LEE4 and LEE5 was maximal at the 3 hour point and remained constant throughout the 5 hour infection. On the other hand, the LEE1 operon that contains the *ler* gene began decreasing at the 3 hour time point. These results show that the Ler activity is less important once the infection has been established, but is crucial in the early stages to remove H-NS repression and to activate the LEE operons. This protein has also been found to have a DNA binding C-terminus homology to the H-NS family of DNA binding proteins.

The Ler protein can be regulated by a large number of factors. Two Ler-regulatory proteins encoded on LEE Island are called GrlA and GrlR act to optimize the LEE1 promoter. A positive regulatory loop formed by GrlA and the Ler protein has been identified, since each activates the expression of the other (Barba, Bustamante et al. 2005). This was confirmed when a *grlA* mutant caused a significant reduction of the expression of the *ler* gene. However, this expression was restored when GlrA was re-introduced. The same results were observed when a *ler* mutant strongly reduced the expression of the *grlA*, but was restored by the introduction of the Ler protein. The GrlR which is expressed from the same operon as GrlA, acts as negative regulator of *ler* expression (Barba, Bustamante et al. 2005; Jobichen, Li et al. 2007). GrlR is involved in down-regulating the GrlA and Ler regulatory loop by binding to GrlA once the Ler protein has reached a threshold, which prevents the accumulation of Ler (Jobichen, Li et al. 2007).

Other proteins can also influence the regulation of the LEE island. The Fis and the integration host factor (IHF), which bind upstream of Ler, are positive regulators of the Ler encoded LEE1 operon (Friedberg, Umanski et al. 1999; Goldberg, Johnson et al. 2001). Interestingly, the IHF did not bind to the LEE2 operon, which verifies that the Ler

protein is the main regulator of the LEE2 to LEE5 operons. A member of the ribosome binding GTPase family called BipA also indirectly regulates the transcription of the LEE Island through Ler (Grant, Farris et al. 2003). A *bipA* mutant demonstrated decreased transcription of the LEE operons including the *ler* gene. The transcription levels could be normalized following introduction of the gene on a plasmid. The Hha protein which regulates the α-hemolysin is also a repressor of the *ler* gene. A *hha* mutant in STEC resulted in a 10-fold increase in the expression of Ler, followed by an increase of LEE1, LEE2, LEE3 and LEE5 expression, and an increase in adherence to HEp-2 cells (Sharma, Carlson et al. 2005). The effect of Hha is believed to be direct, where the repression of *ler* takes place by binding to the *ler* promoter. Two other regulatory pathways called the RcsDCB and the GrvA regulator play a positive role in the expression of Ler (Tobe, Ando et al. 2005).

Regulators of LEE expression can be found on mobile genetic elements. One example is the cluster of genes that possess a second T3SS called T3SS2, found throughout STEC serotypes (Zhang, Chaudhuri et al. 2004). However, the T3SS2 apparatus is not functional, as a result of frameshift mutations occurring over time. This system also bears a resemblance to the *S. Typhimirum* T3SS present on SPI-1 (Lostroh and Lee 2001). Using a microarray, the *etrA* and *eivF* genes located on T3SS2 were found to function as repressors of the transcription of LEE genes (Zhang, Chaudhuri et al. 2004). In addition, *etrA* and *eivF* mutants led to an increase in the expression of LEE genes and the adherence to IECs. This negative effect on the LEE T3SS could result from cross-talk, where the regulators ensure that only one T3SS is being expressed at any given time to avoid the excess use of energy.

Quorum sensing is a cell-to-cell communication mechanism used by bacteria to sense environmental change, and allows them to respond accordingly. Quorum sensing also controls the ability of the bacteria to activate transcription of virulence factors such as the LEE-encoded T3SS (Anand and Griffiths 2003). In the presence of high numbers of pathogenic bacteria, such as microcolonies of STEC on IECs, LEE genes are expressed. This allows bacteria to mount an assault when large numbers are present, overwhelming the host. The quorum sensing *luxS* gene is responsible for the activation of the LEE operons through the regulation of Ler and in the production of the autoinducer 3

(AI-3) (Gasser, Gautier et al. 1955; Barnard and Kibel 1965; Kaplan, Chesney et al. 1975; Walters and Sperandio 2006). A *luxS* mutant caused a reduction of the LEE gene transcription, secretion of effectors and the disruption of A/E lesions. Epinephrine and norepinephrine also play a role in the activation of LEE where the addition of epinephrine enhanced the expression of LEE genes. However, the expression of LEE genes can also be turned on when a low population of bacteria are present (Anand and Griffiths 2003). This effect is due to quorum sensing signals received by non-pathogenic *E. coli* present in the gut.

The regulation of the expression of structural proteins, followed by the expression of the effectors, must also be strictly controlled. A recent study by Deng and colleagues reported that two regulatory proteins SepD and SepL, act as "gatekeepers" to control the switch between the expression of translocon proteins and the expression of effectors (Deng, Li et al. 2005). The mutation of *sepL* and *sepD* genes resulted in the elimination of the secretion of translocators and an increase in the secretion of effectors. Both proteins also appear to interact with one another, and associate with the bacterial membranes. This was noted when a single mutation, or a double mutation, had the same effect [33, 42-44]. Calcium also plays an important role in the switch between translocon proteins and effectors (Deng, Li et al. 2005). In a high calcium environment, such as in the extracellular fluid, translocon proteins are expressed and the translocon is assembled. However, once a pore is formed, the influx of a low calcium concentration switches the production of translocon protein into effector proteins, with the help of SepL and SepD, which can now be secreted into the host cell.

1.3.6 Effectors

1.3.6.1 LEE effectors

Secreted effectors play an important role in the colonization process of enteric pathogens by manipulating host cell pathways and function. The LEE Island which is generally found in the majority of STEC serotypes contains seven (Tir, Map, EspB, EspF, EspH, SepZ and EspG) T3SS secreted effectors (Table 1.3).

Table 1.3 List of Shiga toxin-producing *E. coli* (STEC) effector proteins.

Effector protein	Location	Function	Target site
Tir	LEE	Intimin binding/ A/E lesion formation/ Actin polymerization	Tip of pedestal
Map	LEE	Filopodium formation/ disruption of mitochondrial membrane potential/Disruption of TER	Mitochondria/ Actin
EspF	LEE	Induction of cell death/ disruption of mitochondrial membrane potential/Disruption of TER/ disruption of tight junctions/ inhibition of phagocytosis/ microvilli effacement/ apoptosis	Mitochondria/ Apical and lateral membrane
EspG	LEE	Destruction of microtubule network/ Actin stress fiber formation	Pedestal/ co-localization with tubulin
EspB	LEE	Cytoskeleton modulation/ Pore formation	Bacterial attachment site/ plasma membrane
EspZ	LEE	Unknown	Unknown
EspH	LEE	Elongation of pedestals/ cytoskeleton disruption	Pedestal structure
NleA	CP-933P prophage	Interference of protein secretion	Golgi
NleB	OI-122	Unknown	Unknown
NleC	CP-933K prophage	Unknown	Unknown
NleD	CP-933K prophage	Unknown	Unknown
NleE	OI-122	Unknown	Unknown
NleF	OI-71	Unknown	Unknown
NleG (14 homologs)		Unknown	Unknown
NleH1	CP-933K prophage	Pro-inflammatory	Unknown
NleH2	CP-933K prophage	Pro-inflammatory	Unknown
Cif	Lamboid prophage	Cyclomodulin activity	Unknown
EspJ	CP-933U prophage	Regulation of clearance/ / inhibition of phagocytosis	Unknown
TccP	CP-933U prophage	A/E lesion formation/ Actin polymerization	Tip of pedestal
TccP2	CP-933M prophage	A/E lesion formation/ Actin polymerization	Tip of pedestal

1.3.6.1.1 Tir

One of the most important and best-studied LEE effectors is the translocated intimin receptor, Tir. The study of Tir began with the identification of the STEC outer membrane adhesin called intimin (Jerse, Yu et al. 1990). Initially, it was assumed that intimin bound to a host 90 kDa host protein called Hp90. However, Kenny and colleagues discovered that the Hp90 was actually a LEE encoded T3SS effector protein, eventually re-named Tir (Kenny, DeVinney et al. 1997). This surprising ability of a bacterial pathogen to carry its own receptor is a trait only observed in A/E pathogens.

Once Tir is translocated through the T3SS, it localizes in the plasma membrane of the eukaryotic cell (Kenny, DeVinney et al. 1997; Deibel, Kramer et al. 1998). This protein contains two trans-membrane domains, which form a surface-exposed extracellular loop responsible for binding intimin, while the N and C termini are buried within the eukaryotic cell (de Grado, Abe et al. 1999; Hartland, Batchelor et al. 1999; Kenny 1999). This extracellular loop is 104 amino acids long and is critical for intimin binding and pedestal formation (de Grado, Abe et al. 1999). Both Tir and intimin proteins are dimers, allowing each intimin monomer to bind to a monomer of the Tir protein, resulting in an intimate interaction between the bacterium and the host cell (Luo, Frey et al. 2000). The intracellular N and C termini of the Tir protein can recruit and interact with a number of intracellular host and bacterial proteins. These interactions result in actin polymerization and pedestal formation observed with a typical STEC infection.

The actin polymerization involved in the formation of the pedestal is dependent on the C terminus of Tir protein (Campellone, Rankin et al. 2004). Although the N terminus interacts with a number of host proteins, the deletion of this terminus still allows formation of A/E lesions (Campellone, Rankin et al. 2004). Following attachment of Tir to intimin, actin polymerization is triggered by the bacterial T3SS secreted protein called TccP, also referred to as $EspF_U$ (Garmendia, Frankel et al. 2005; Frankel and Phillips 2008). This protein is capable of recruiting and activating the Neural Wiskott-Aldrich syndrome protein (N-WASP), which in turn triggers the actin-nucleating Arp2/3 complex, resulting in actin polymerization (Figure 1.6). A number of other host proteins such as cortactin and Gbr2 are also recruited to assist with the

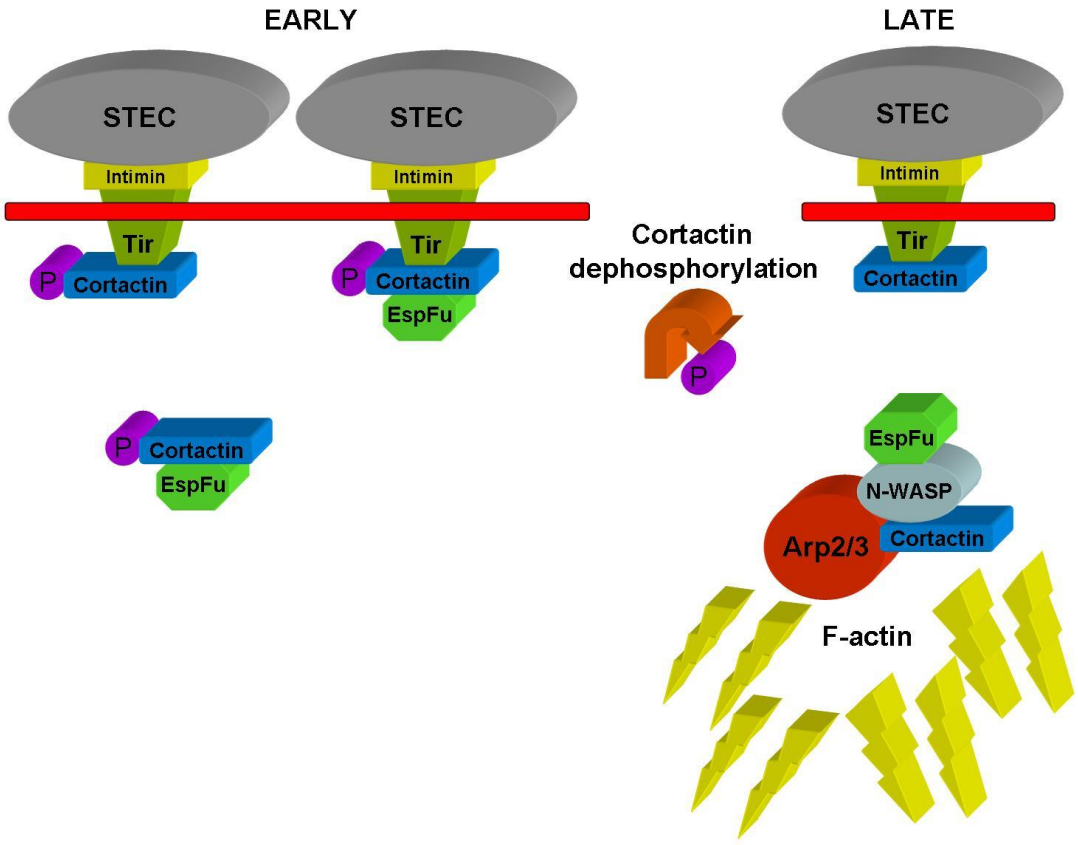


Figure 1.6 Model for protein interaction during the formation of STEC pedestal. During the early stages of pedestal formation STEC attaches to the host cell through the Tir-intimin interaction. The tyrosine phosphorylated host protein cortactin binds Tir and TccP which accumulates around the attached bacterium. Cortactin may also interact with both proteins at the same time. During the later stages, cortactin dephosphorylation releases cortactin and TccP from Tir. The TccP protein then recruits and activates the neurol Wiskott-Aldrich syndrome protein (N-WASP), which in turn triggers the actin nucleating Arp2/3 complex, resulting in actin polymerization. STEC can be referred as Enterohemorrgic *E .coli* (EHEC). TccP is also referred to as $EspF_U$. Diagram adapted and modified from (Cantarelli, Kodama et al. 2007).

amplification of the signal (Goosney, DeVinney et al. 2001; Cantarelli, Takahashi et al. 2002). The cortactin protein directly binds to Tir and the TccP protein, where it links the bacterial proteins to the actin polymerization, and helps guide pedestal formation through closely controlled tyrosine phosphorylation and dephosphorylation of cortactin proline-rich domains (Figure 1.6) (Cantarelli, Kodama et al. 2007). Several other Tir-independent host proteins such as calpactin, CD44, ezrin, talin, gelsolin and tropomyosin localize to the site of bacterial attachment (Goosney, DeVinney et al. 2001). The role of Tir within EPEC and STEC is almost identical, where the main difference is observed were STEC uses TccP to activate N-WASP, while EPEC recruits the host Nck protein to employ the N-WASP/Arp2/3 complex (Gruenheid, DeVinney et al. 2001). However, recently, *in vivo* studies have identified a common actin polymerization pathway used by both STEC and EPEC strains. A number of non-O157 STEC serotypes, sorbitol-fermenting STEC O157, as well as EPEC strains, possess a gene that expressed a protein with a 69% homology to the STEC TccP protein, called TccP2 (Ogura, Ooka et al. 2007). Over 95% of non-O157 STEC serotypes that possess the *tccP2* gene, 90% also contain a Tir protein that can be tyrosine phosphorylated and trigger the Nck actin polymerization pathway. Interestingly, this protein functionally complements a STEC O157 *tccP* mutant, when the gene is introduced on a plasmid.

1.3.6.1.2 Map

The Mitochondrial associated protein (Map) was given its name because of its ability to target and disrupt the mitochondria via its N-terminal signal sequence (Kenny and Jepson 2000). This disruption occurs by interfering with the ability of the mitochondria to maintain membrane potential, which causes mitochondrial swelling, the formation of distorted mitochondria and eventual organelle damage (Kenny and Jepson 2000; Kenny 2002). The Map protein is a multifunctional effector that can play a number of roles during STEC infection, such as the disruption of barrier function and tight junction structure (Dean and Kenny 2004). This protein is also involved in cytoskeletal rearrangement by inducing Cdc42-dependent filopodium-like structures, which is not dependent on mitochondrial targeting (Kenny, Ellis et al. 2002).

Using the *C. rodentium* mouse model, a *map* mutant induced lower levels of hyperplasia while efficiently colonizing the mouse (Mundy, Petrovska et al. 2004). In a competition infection between the mutant and the wildtype strain, the mutant initially colonized the host at low doses, but was outcompeted by the wildtype strain by day 9. These results confirm that the Map protein gives STEC the ability to outcompete a Map-negative strain in a competitive environment. In addition Map may also play a role in altering immunological responses.

Interestingly, both Tir and Map proteins interact with a 15 kDa cytoplasmic protein called the chaperone for *E. coli* secretion of Tir (CesT) (Elliott, Hutcheson et al. 1999; Delahay, Shaw et al. 2002; Creasey, Delahay et al. 2003). This interaction which occurs at the amino acid terminus, is not absolutely necessary for secretion, where both proteins, although nearly abolished, could still be secreted. However, a *cesT* mutant was unable to form A/E lesions (Elliott, Hutcheson et al. 1999). These results demonstrate that CesT is a chaperone required for the translocation of both Map and Tir secreted proteins.

1.3.6.1.3 EspF

The EspF protein is involved in the disruption of barrier function and tight junction structure, as well as in the increase of membrane permeability and the distribution of the tight junction protein called occludin (McNamara, Koutsouris et al. 2001; Dean and Kenny 2004). The disturbance of the barrier function involves the coordinated action of both Map and EspF, where individually *map* or *espF* mutants showed reduced disruption compared to the wildtype (McNamara, Koutsouris et al. 2001). Surprisingly, an intimin mutant was unable to disrupt barrier function, implying that the EspF and Map affect may be dependent on the intimin protein or on attachment.

The EspF protein is transported to the mitochondria, where it releases the mitochondrial cytochrome C protein, and helps in the cleavage of caspases 9 and 3, both implicated in cell death (Nougayrede and Donnenberg 2004; Nagai, Abe et al. 2005). The EspF protein has also been suggested to play a role in other virulence aspects such as apoptosis and the inhibition of the uptake by macrophages (Crane, McNamara et al. 2001; Marches, Covarelli et al. 2008). In addition, *in vitro* experiments using organ

cultures demonstrated that an *espF* mutant displayed A/E lesions with non-effaced microvilli still uniform in length. This implies that EspF could play a role in the re-modeling of the brush border microvilli (Shaw, Cleary et al. 2005).

When an *espF* mutant was tested in an *C. rodentium* mouse model, a significant decrease in virulence was observed, with a reduction in mortality, colon weight and intestinal mucosal layer thickness (Nagai, Abe et al. 2005). These results demonstrate that the abilityv of EspF to be transported to the mitochondria plays an important role in the colonization of the gastrointestinal area.

1.3.6.1.4 EspG

The LEE-encoded EspG protein is involved in triggering actin stress fiber formation and the destruction of the microtubule networks underneath adherent bacteria, resulting in an increase in paracellular permeability (Matsuzawa, Kuwae et al. 2004). Using Swiss 3T3 mouse cells in *in vitro* experiments, EspG was shown to interact with tubulins causing destabilization of microtubules. Similar results were confirmed with Caco-2 cells, were the cytoskeleton was disrupted through microtubule depletion due to tubulin binding (Shaw, Smollett et al. 2005). This microtubule destabilization involves the release of the attached microtubule GEF-H1 protein, followed by the activation of the RhoA-ROCK signalling pathway (Matsuzawa, Kuwae et al. 2004). A sequence homology exists between EspG and the VirA effector of *S. flexneri*, which is also involved in the interaction of tubules causing microtubule instability (Yoshida, Katayama et al. 2002). Using the *C. rodentium* mouse model, infection with an EspG mutant resulted in a significant reduction in the ability of STEC to colonize the gastrointestinal tract, and to cause colonic hyperplasia (Hardwidge, Deng et al. 2005).

1.3.6.1.5 EspB

The EspB protein contributes to the formation and secretion of the T3SS translocon by assisting in the development of a surface pore on IECs (Fivaz and van der Goot 1999; Warawa, Finlay et al. 1999). However, EspB also plays a role as a bacterial effector. This protein can be transported to the host cell cytoplasm, where it localizes at the site of bacterial attachment and participates in the re-organization of actin structures

(Taylor, O'Connell et al. 1998; Taylor, Luther et al. 1999). Epithelial cells transfected with EspB displayed altered morphology where the number of stress fibers was reduced (Taylor, Luther et al. 1999). It is proposed that EspB functions as a cytoskeleton toxin by releasing monomeric actin for the re-organization of the filamentous actin under pedestals. This was suggested since transfected cells were able to concentrate high amounts of actin underneath bacteria, without stress fibers. The role of EspB in the modulation of the actin cytoskeleton was further confirmed when Kodoma and colleagues identified that EspB was capable of recruiting and interacting with the cytoskeleton associated protein called α-catenin, essential for A/E lesion formation (Kodama, Akeda et al. 2002).

1.3.6.1.6 EspZ

The EspZ protein localizes underneath the bacterial attachment site, around the Tir protein (Kanack, Crawford et al. 2005). Using the *C. rodentium* mouse model, EspZ was shown to be required for full virulence and pathogenesis during infection, where an *espZ* mutant did not cause development of the characteristic colonic hyperplasia. A significant reduction in mortality was also observed. However, the precise role and function of the EspZ effector is yet to be determined.

1.3.6.1.7 EspH

The LEE-encoded EspH protein is translocated through the T3SS, where it localizes at the pedestal structure underneath the adherent bacterium (Tu, Nisan et al. 2003; Deng, Puente et al. 2004). This protein represses filopodium formation, and is involved in controlling pedestal length, where the over-expression of EspH led to the elongation of flat pedestals. EspH also disrupted COS cells actin polymerization by acting as Rho, Rac and Cdc42 inhibitors (Ben-Ami, Ozeri et al. 1998). However, when an *espH* mutant was tested for virulence in the *C. rodentium* mouse model, the strain was only slightly attenuated (Deng, Puente et al. 2004). Overall, the EspH protein is involved in the repression of infected host cells and appears to play a role as a modulator of the actin cytoskeleton (Tu, Nisan et al. 2003).

1.3.6.2 Non-LEE effectors

1.3.6.2.1 NleA

In 2004, through the use of proteomics, the first non-LEE encoded (NLE) effector called NleA was identified (Table 1.3). The CP-933P prophage-encoded NleA effector, also known as EspI, is secreted through the T3SS and is transported to the Golgi apparatus (Gruenheid, Sekirov et al. 2004). Once it reaches the Golgi, it co-localizes with the mannoside II and Golgin-97 host proteins. NleA was also found to bind a number of host proteins through a PDZ binding motif. Using a yeast two-hybrid system, and a PDZ-domain protein array overlay, NleA interacted with a total of 15 host proteins (Lee, Kelly et al. 2008). These proteins are involved in the formation of tight junctions, membrane integrity, ion channels, and protein signalling to the host cell membrane (Albrecht and Froehner 2002; Lee, Kelly et al. 2008).

When a *C. rodentium nleA* mutant was tested in the mouse model, it was found to be non-lethal and the mice did not develop the typical colonic hyperplasia (Gruenheid, Sekirov et al. 2004; Kelly, Hart et al. 2006). Although NleA plays a role in colonization, it is not required for the formation of A/E lesions. A recent study identified that 86% of disease-causing STEC strains contained the *nleA* gene (Mundy, Jenkins et al. 2004). In addition, using statistical analysis, *nleA* was also found to be present in STEC strains most commonly associated with severe human disease.

1.3.6.2.2 NleB-NleH

Apart from NleA, a number of other effectors from the Nle group have been identified (Table 1.3) (Roe, Tysall et al. 2007). However, their roles and functions are not yet fully understood. The OI-122 *nleB* gene, and the immediate upstream *nleE* gene, are secreted through the T3SS and are involved in colonization and disease as described (Tobe, Beatson et al. 2006; Wickham, Lupp et al. 2006; Wickham, Lupp et al. 2007). The *nleB* gene is present in 100% of STEC O157:H7 and 69% of non-O157 disease causing strains. In addition, when a *C. rodentium nleB* mutant was tested *in vivo*, it required an infectious dose 285-fold higher than the wildtype strain, while demonstrating a reduction in colonization and in colonic hyperplasia of mice. These results have established that the

NleB protein is a vital component of STEC pathogenesis (Kelly, Hart et al. 2006; Wickham, Lupp et al. 2006). The NleE protein has also been shown to be essential for the full virulence of STEC (Wickham, Lupp et al. 2007). Non-O157 strains that possess the *nleE* gene are 28 times more likely to be involved in outbreaks, and four times more probable to cause HUS (Wickham, Lupp et al. 2006). When tested in the mouse *C. rodentium* model, the *nleE* gene was required for full colonization and introduction of disease.

Although NleC and NleD are transported by the LEE-encoded T3SS, deletion mutants are still able to infect both calves and lambs and cause A/E lesions (Marches, Wiles et al. 2005; Tobe, Beatson et al. 2006). These results suggest that neither protein is required for STEC colonization, but further studies are necessary to fully understand their role in STEC infection. The NleF protein, which is found on OI-71, is also secreted through the T3SS into the host cytoplasm (Tobe, Beatson et al. 2006; Echtenkamp, Deng et al. 2008). This protein is believed to interact with other effectors in order to decrease the host response to infection. Although, no role was observed in bacterial adherence, cytoskeleton rearrangement, or host protein interaction, a competitive co-infection of mice demonstrated that a *C. rodentium nleF* mutant had reduced levels of colonization. An infection study of the terminal rectum and piglet colon with an *nleF* mutant also demonstrated reduced colonization compared to the wildtype (Echtenkamp, Deng et al. 2008). Interestingly, seropathotype classifications completed on STEC strains have shown that NleF is affiliated with seropathotype A, which is the group most likely to cause severe human disease.

A family of 14 effectors belonging to the NleG family were recently identified in STEC O157 (Tobe, Beatson et al. 2006). These effectors were discovered through a short sequence peptide which, after a TBLASTN search, revealed several NleG homologs. The majority of these proteins have also been shown to be secreted through the T3SS. Although their role in STEC infection is unknown, the presence of conserved sequence patches of histidine, cysteine and aspartate amino acid residues, suggest that they may have conserved enzymatic activity (Mukherjee, Keitany et al. 2006; Tobe, Beatson et al. 2006).

Another important non-LEE effector, which appears to have a host specific response, is NleH (Garcia-Angulo, Deng et al. 2008). Shiga toxin-producing *E. coli* serotypes have two nearly identical copies of the *nleH* gene (*nleH1* and *nleH2*), while *C. rodentium* only contains one (Tobe, Beatson et al. 2006; Garcia-Angulo, Deng et al. 2008). Infection of lambs or calves with an *nleH* mutant showed varying results (Hemrajani, Marches et al. 2008). In an oral challenge of lambs, a Δ*nleH1*Δ*nleH2* mutant out-competed a wildtype strain, while oral challenge of calves resulted in a reduction of shedding by the mutant. In a mouse model, the level of activation of NF-κB and TNF-α were significantly reduced compared with the wildtype (Hemrajani, Marches et al. 2008). These results suggest that NleH protein plays a role in the activation of NF-κB, which is responsible for an increase of inflammatory cytokines. However, to fully understand its functional properties, further studies must be completed.

1.3.6.2.3 Other non-LEE encoded secreted proteins

The first STEC prophage effector to be identified was the cycle inhibiting factor (Cif) (Table 1.3). This effector, which is located on a lamboid prophage, is secreted through the T3SS, and is a member of the cyclomodulin family of proteins that target the host cell cycle (Marches, Ledger et al. 2003). Using HeLa cells, the Cif protein was shown to have irreversible cytopathic effect involving the recruitment of focal adhesins, congregation of stress fibers, and the arrest of the cell cycle G_2/M phase transition through the accumulation of the inactive Cdk1 protein (Marches, Ledger et al. 2003; Samba-Louaka, Nougayrede et al. 2008). The Cif protein can also block the G_1/S phase transition by stabilizing and allowing the accumulation of the cyclin dependent kinase inhibitors $p21^{waf1}$ and $p27^{kip1}$ (Samba-Louaka, Nougayrede et al. 2008). In addition, the infection of HeLa cells with *cif* mutant was unable to block mitosis but did affect the formation of A/E lesions (Marches, Ledger et al. 2003).

Another important effector located on a CP-933U prophage is the *espJ* gene directly upstream of *tccP* (Table 1.3). The infection of mice with an *espJ* mutant resulted in prolonged colonization and the inability of mice to clear the infection (Dahan, Wiles et al. 2005). Similar results were observed with lambs when challenged with both the wildtype and the mutant strain. Although colonization levels were identical, the mutant

strain persisted longer for a period of 18 days. Surprisingly, this effector appears to be involved in pathogen clearance from the host gastrointestinal tract. Recent studies have also shown that EspJ is involved in the inhibition of phagocytosis by blocking both FcγR- and CR2-mediated phagocytosis, by possibly interacting with a host complex downstream (Marches, Covarelli et al. 2008).

The TccP effector is present on the CP-933U prophage (Table 1.3). As described earlier, this protein is involved in the binding of Tir and host cortactin protein where it activates the N-WASP protein that recruits and binds the Arp2/3 complex allowing actin polymerization (Figure 1.6) (Garmendia, Frankel et al. 2005; Cantarelli, Kodama et al. 2007; Frankel and Phillips 2008). Recently, a second T3SS secreted effector called Tccp2 was identified and is involved in triggering the Nck actin polymerization pathway (Ogura, Ooka et al. 2007).

With the identification of the first non-LEE effector NleA, the study of STEC pathogenesis began to focus on other possible secreted effectors positioned outside of the LEE Island. Using bioinformatics and other experimental approaches, an important study in 2006 by Tobe and colleagues identified a new set of 28 non-LEE effectors, which are secreted through the T3SS, and are located in various lamboid prophages and pathogenicity islands (Tobe, Beatson et al. 2006). Until this point only a small group of proven non-LEE effectors had been discovered in *C. rodentium* (Deng, Puente et al. 2004; Gruenheid, Sekirov et al. 2004; Marches, Wiles et al. 2005). However, at present, the role of the majority of these newly identified non-LEE effectors remains unknown.

1.4 Host response

1.4.1 Host response to enteric pathogens

Intestinal mucosal surfaces are constantly exposed to large amounts of both commensal and pathogenic bacteria. It is estimated that the human colon contains approximately 10^{14} bacteria (Hooper, Midtvedt et al. 2002) representing over 1000 different bacterial species. These bacteria include both resident microflora as well as pathogenic bacteria that are in transit throughout the bowel. Early recognition and

subsequent neutralization of these bacteria is therefore crucial for prevention of gastrointestinal diseases.

The gastrointestinal-associated lymphoid tissues (GALT) represent the largest immune compartment within the body. The GALT recognize and prevent pathogens from invading the mucosal tissues and tolerate commensal organisms that are required for effective food digestion and processing (Figure 1.7). To this end, the mucosal surfaces are equipped with an effective surveillance system that relies on both innate and acquired immune mechanisms involving a variety of effector molecules and immune cells (Athman and Philpott 2004). The main function of the GALT is to distinguish between commensal and pathogenic flora resulting in either oral tolerance or active immune responses. The GALT are equipped with a variety of receptors and effector molecules that enable recognition of conserved pathogen-associated molecular patterns (PAMPs). Pattern recognition receptors (PRRs), located primarily on IECs, include TLRs, NOD proteins, mannose receptors, and C-type lectins (Figure 1.7). Stimulation of PRRs with bacterial products, such as LPS or pro-inflammatory cytokines, such as IL-1β and TNF-α, trigger signalling cascades that lead to inducible nitric oxide synthase (iNOS) transcription. This pathway results in the activation of p38 mitogen-activated protein kinase (MAPK), NF-κB and Janus-activated kinase–signal transducer and activator of transcription–interferon regulatory factor 1 (JAK–STAT–IRF1) pathways (Kamijo, Harada et al. 1994; Xie, Kashiwabara et al. 1994). In addition, upregulation of phagocytic co-stimulatory molecules leads to T cell activation.

Intestinal epithelial cells possess TLRs such as TLR 2, 3, 4, 5, 7/8 and 9 (Lundin, Bok et al. 2008) that contain three major domains. The amino-terminal domain consists of a series of leucine rich repeat (LRRs) motifs followed by a transmembrane domain and a C-terminal Toll/IL-1 receptor (TIR) domain. The LRR domains of TLR2 and TLR5 have been shown to bind peptidoglycan (PG) and flagellin (Iwaki, Mitsuzawa et al. 2002), respectively. Following ligand recognition and binding by the LRR domain, a signal is transduced across the membrane to the TIR domain, which activates associated adaptor proteins and subsequent signal transduction cascades. TLR2 is functionally associated with TLR1 and/or TLR6. The TLR2/TLR1 heterodimer recognizes triacylated lipopeptides, whereas the TLR2/TLR6 recognizes lipoproteins and lipoteichoic acid

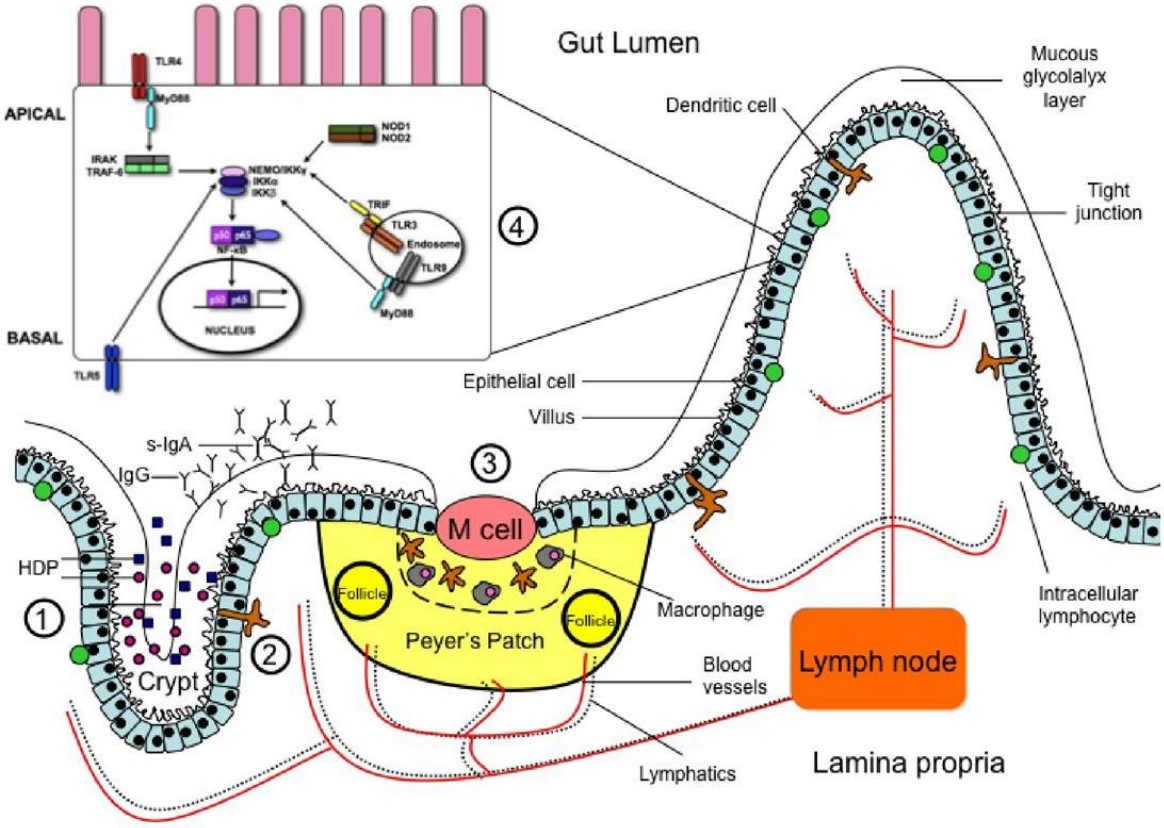


Figure 1.7 Inductive and effector sites in intestinal mucosa. The intestinal mucosa includes the epithelial layer, associated brush border, tight junctions, and mucosa. (1) Paneth cells at the intestinal crypts secrete host defense peptides (HDPs), which contribute to local immunity. (2) Dendritic cells extend processes into the lumen to sample antigen. (3) An M cell, a gateway for bacterial entry, within the Peyer's patch is shown. (4) The inset Figure shows components involved in host recognition of pathogen-associated molecular patterns (PAMPs) via TLR4 (apical), TLR5 (basal) or intracellular NOD, TLR3 and TLR9. Ligand binding culminates in MyD88-dependent activation of NF-κB and expression of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α) and IL-8.

(LTA), found in the PG layer of Gram-positive bacteria (Schwandner, Dziarski et al. 1999; Yoshimura, Lien et al. 1999). Binding of LPS to TLR4 and CD14 results in the recruitment of MyD88 (myeloid differentiation primary response gene 88) through the interaction of TIR-homology domains in TLR4 and MyD88, whereas the amino-terminal death domain of MyD88 interacts with IL-1-receptor-associated kinase (IRAK) (Silverman and Maniatis 2001). Subsequent to the activation of IRAK, another adaptor protein, TNF-receptor-associated factor 6 (TRAF6) is phosphorylated and recruited to IRAK to activate the NF-κB pathway. Flagellin, a bacterial virulence factor, is recognized by TLR5 present on the basolateral side of the epithelial cell (Hayashi, Smith et al. 2001) whereas TLR9 is present in the endosome and recognizes unmethylated bacterial cytosine–phosphate–guanosine (CpG) motifs (Hemmi, Takeuchi et al. 2000). Another type of important PRR is NOD. NOD1 and NOD2 are intracellular receptors for PGN (Girardin, Boneca et al. 2003; Girardin, Boneca et al. 2003), whereby NOD1 detects PGN from Gram-negative bacteria, and NOD2 detects PGN from both Gram-negative and Gram-positive bacteria (Inohara, Koseki et al. 1999; Girardin, Boneca et al. 2003).

In response to stimulation, IECs secrete effector molecules including antimicrobial peptides, cytokines, and chemokines that recruit polymorphonuclear cells, macrophages, dendritic cells (DCs), and lymphocytes which are capable of immune modulation and killing pathogens. Local cytokine expression also results in the activation and maturation of these cells. For example, granulocyte macrophage-colony stimulating factor (GM-CSF), IL-6, and TNF-α promote macrophage activation, proliferation, and additional cytokine secretion, resulting in the engulfment and destruction of pathogens (Eckmann and Kagnoff 2001). Secretion of TNF-α, IFN-γ, IL-1α, IL-12, IL-18 and IL-15 further enhances the pro-inflammatory response, which alternatively can be suppressed by IL-4 and IL-10 (Eckmann and Kagnoff 2001). Recruitment of immune cells into the subepithelial region is dependent on the recognition of PAMPs such as flagellin or PGN by infected IECs (Mrsny, Gewirtz et al. 2004).

Although IECs respond poorly to resident microflora (Otte, Cario et al. 2004), they influence the responsiveness of DCs towards the microflora. The local microenvironment manipulates the priming ability of DCs to different subsets of T cells, including T helper 2 (T_H2) cells (Iwasaki and Kelsall 1999) and T regulatory (Treg) cells

(Kelsall and Leon 2005). Recently, thymic stromal lymphopoietin (TSLP) has been shown to be constitutively released by IECs and needed for IEC-DC crosstalk (Zeuthen, Fink et al. 2008). How aspects of the microflora influence the production of IEC-derived factors is unknown, but it is possible that the microflora influences the underlying DC through the IECs. IEC-DC crosstalk represents an important ability of the immune system to respond to alterations in gut homeostasis. By priming antigen-specific naïve CD4+ and CD8+ T cells, DCs are critical for initiating adaptive immunity in response to infection by pathogenic bacteria (Rescigno 2002). Dendritic cells residing in the intestinal mucosa sense bacterial PAMPs, mature, and migrate into the mediastinal lymph node (MLN), where antigens are presented to T and B lymphocytes. B cells undergo differentiation, migrate to the lamina propria (LP), and secrete IgA locally (Means, Hayashi et al. 2003).

The innate defense against enteric pathogens includes host defense peptides (HDPs), lysozyme, phospholipase A2, and α1-antitrypsin (Lehrer and Ganz 2002). Host defense peptides are small cationic polypeptides that mediate host defense in mammals, plants and insects (Boman 2003) and have direct, broad-spectrum antimicrobial activity through direct killing of bacteria by membrane permeabilization (Yeaman and Yount 2003). Immunostimulatory functions include induction of chemotaxis of immature dendritic cells and T cells, activation of antigen-presenting cells (APC), increased glucocorticoid production, macrophage phagocytosis, mast cell degranulation, complement activation, and IL-8 production by epithelial cells (Biragyn, Belyakov et al. 2002).

One class of HDPs are defensins, which are divided into α-, β- and θ-defensins, based on structural features (Lehrer and Ganz 1999). Livestock have been shown to express a wide variety of β-defensins (Selsted, Tang et al. 1996) and porcine defensins have been shown to be microbicial against intestinal pathogens (Veldhuizen, Rijnders et al. 2007). Cathelicidins are another class of HDPs which possess broad-spectrum activity against bacteria and fungi, including clinical isolates resistant to antibiotics (Gennaro and Zanetti 2000). The peptides have been demonstrated to bind to LPS and provide protection in animal models of endotoxemia (Larrick, Hirata et al. 1994) and bacterial infection (Brogden, Kalfa et al. 2001).

Invading pathogens must also overcome the mucus produced by goblet cells. Intestinal goblet cells are highly polarized secretory cells present throughout the intestinal tract which increase in number from the esophagus to the rectum (Specian and Oliver 1991). Mucus, the gel layer overlying epithelial cells, is required for the lubrication of the intestinal surface. This layer limits the passage of luminal molecules into the mucosa, acts as a defensive barrier against enteric pathogens (Deplancke and Gaskins 2001), and as a substrate for colonization of commensal flora (Sonnenburg, Xu et al. 2005) (Figure 1.7). Goblet cells synthesize mucins, such as MUC2, a glycoprotein present in the mucus (Moncada, Kammanadiminti et al. 2003), which inhibit the adherence of enteric pathogens.

Intestinal macrophages (IM) which also play an important role against enteric pathogens, are located in the sub-epithelial dome region of PP, with the small intestine containing fewer mucosal macrophages than the colon (Lee, Starkey et al. 1985). Intestinal macrophages interact with luminal bacteria and detect microbes and microbial products that cross the epithelium and clear local pathogens without prior activation (Macpherson and Uhr 2004). In addition to lacking TLRs, IMs fail to express receptors present on other macrophage populations needed to detect and respond to bacteria. One example is the IgA-specific Fc high affinity receptor (FcαR), which interacts with IgA (Smith, Smythies et al. 2001). IgA is the most abundant antibody isotype in the GALT and IgA-mediated phagocytosis can induce a respiratory burst in IMs (Babior 1984). Intestinal macrophages also lack the FcγR1 and FcγRIII receptors for IgG, as well as the CR3 and CR4 complement receptors (Smythies, Sellers et al. 2005). The absence of these receptors may contribute to their lack of pro-inflammatory responses.

1.4.2 Host response to STEC

1.4.2.1 Innate response

The innate response plays an important role in the rapid defence against STEC infections. This initial response is non-specific, and protects the host until it is capable of developing the more specialized adaptive response. The initial line of defense is the presence of the mucus barrier. Goblet cells synthesize mucins such as MUC2, a

glycoprotein (Moncada, Kammanadiminti et al. 2003) which inhibits the adherence of enteric pathogens, such as STEC (Mack and Sherman 1991). The normal flora found within the gut can also help reduce STEC O157 infection by priming the immune system. The activation of MUC2 and IECs by probiotic bacteria such as *Lactobacillus acidophilus* was found to protect against STEC infection (Kim, Kim et al. 2008). An *in vitro* study using *L. acidophilus* bacteria and cell extracts, resulted in the production of MUC2 and increased mRNA levels of IL-1β, TNF-α and IL-8 (Kim, Kim et al. 2008). This increase resulted in the inhibition of attachment of STEC O157 to the epithelial cell layer.

Phagocytosis along with macrophage signalling are common methods used by the host to combat invading enteric pathogens. Phagocytosis involves the uptake of large foreign material by neutrophils, macrophages and dendritic cells. However, STEC with a functional Type III apparatus can limit its uptake by inhibiting the phosphatidylinositol 3-kinase (PI3K)-dependent F-actin rearrangements required for uptake (Celli, Olivier et al. 2001). The ability of STEC and EPEC to control this uptake by phagocytes is due to effectors EspF and EspJ, which are responsible for the inhibition of FcγR- and CR3 mediated phagocytosis (Marches, Covarelli et al. 2008).

Initial contact between a host and an enteric pathogen leads to induction of inflammation through TLRs as described. A single bacterium is able to activate multiple TLRs such as TLR4 by LPS, TLR5 by flagellin, or TLR2 which recognizes PG (Lundin, Bok et al. 2008). Signalling via MyD88 is necessary for the recruitment of macrophages for bacterial phagocytosis and the re-establishment of the epithelial layer. A study using MyD88-/- mice reported that infection with STEC O157 led to substantially more disease and increased shedding compared to the wildtype (Calderon Toledo, Rogers et al. 2008). Numerous studies have also been completed on A/E pathogens using the *C. rodentium* model. A separate study using MyD88-/- mice infected with *C. rodentium* demonstrated similar results with increased shedding and severe colonic pathology (Lebeis, Bommarius et al. 2007). However, these mice also had delayed neutrophil recruitment and mortality. The increase in pathological and haematological changes with MyD88-/- mice, imply that the exclusion of the innate response during STEC infection leads to an increase in disease.

Mast cells are implicated in the host inflammatory response by secreting IFN-α and by recruiting neutrophils, which are involved in bacterial clearance (Malaviya, Ikeda et al. 1996). Surprisingly, the use of mast cell deficient mice infected with *C. rodentium*, resulted in severe inflammation, increased cytokine production, tissue damage, bacteraemia and increased mortality compared to wildtype (Wei, Hilliard et al. 2005). These results show that mast cells play a protective role by preventing bacteraemia and sepsis, by facilitating the repair of IECs used for bacterial entry, and by destroying pathogens (such as *C. rodentium*) with the release of antimicrobial factors.

A consequence of STEC infection is the initial up-regulation of signalling via NF-κB, p38 and ERK MAP kinases, resulting in the increase of IL-8 expression and neutrophil migration (Savkovic, Koutsouris et al. 1997). However, after prolonged infection, STEC suppress this activation, likely via the secreted EspB effector (Hauf and Chakraborty 2003). The delayed suppression is consistent with the sequence of events, where initially the bacterium adheres to the host cell, followed by the assembly of the secretion apparatus, which triggers the secretion of effectors. STEC can also down-regulate the expression of iNOS (Maresca, Miller et al. 2005), which occurs through the decrease of STAT-1 activation (Vareille, Rannou et al. 2008). Interestingly, the nitric oxide (NO) response has been shown to reduce the Stx synthesis and its release in the gut (Vareille, de Sablet et al. 2007).

The clearance of A/E pathogens such as STEC involves a combination of protective and destructive elements of the innate immune system (Lebeis, Sherman et al. 2008). The infection of mice with *C. rodentium* is thought to induce a strong mucosal Th1 response by macrophages and T cells in the area, which induce the production of IL-12, IFN-γ and TNF-α. This induction is partly responsible for the crypt hyperplasia and acute colitis seen in these animals (Higgins, Frankel et al. 1999; Higgins, Frankel et al. 1999). However, crypt hyperplasia, which is also observed with STEC and EPEC, could benefit the host. The amount of carefully controlled epithelial cells produced by the colonic crypts is disrupted by *C. rodentium,* which results in an increase in crypt height. This increase causes the rapid sloughing of IEC into the lumen while the bacteria are still attached, which could help with the clearance of the bacterial infection (Vallance, Deng et al. 2002).

Interleukin-1 receptor (IL-1R) is also important in resolving *C. rodentium* infection in mice (Lebeis, Powell et al. 2009). Infected IL-1R-/- mice had reduced levels of IL-6 and IFN-γ, and increased intestinal damage, including gangrenous mucosal necrosis, colonic bleeding and mortality. The IL-1R is involved in inducing IL-6 and IFN-γ levels, which when combined are important in resolving enteric infections. Overall, these results demonstrate that an innate response plays a critical role during the initial infection, by holding the bacteria within the colon, and destroying any that escape the region.

1.4.2.2 Adaptive response

The adaptive response of a host is a delayed and specific cellular response. At present, the majority of studies done on the adaptive response against A/E pathogens have been completed using the *C. rodentium* model. Initial studies with mice lacking acquired immunity, such as RAG-1- knockout mice deficient in mature T and B lymphocytes, demonstrated that challenge with *C. rodentium* does not allow the clearance of infection, but instead results in heavy shedding with high mortality. In contrast, infection of wildtype mice leads to a inflammatory response followed by crypt hyperplasia, with full recovery within 3 weeks (Vallance, Deng et al. 2002). The RAG1 knockout mice initially showed a similar response to the wildtype, but quickly diverged within the first 2 weeks. This illustrates that the innate system produces a reaction that can hold a pathogen at bay, while the adaptive response may be necessary to resolve the infection. Interestingly, the IFN-γ expression levels differed significantly between the wildtype and the RAG-1- knockout mice. These levels were raised substantially in the wildtype mice, while in the RAG-1- knockout mice, IFN-γ expression dropped below uninfected levels. The expression of IFN-γ has been shown to be involved in the development of crypt hyperplasia (Artis, Potten et al. 1999).

Other researchers demonstrated that mice with depleted CD4+ T cells showed greater shedding, and did not clear the *C. rodentium* infection, from both mucosal and systemic tissues. It was suggested that the reduction in CD4+ T cells decreased their ability to assist B cells causing a depleted antibody response. The use of μMT mice, which have no IgG+ or IgM+ B cells were shown to be extremely vulnerable to *C.*

rodentium infection (Macpherson, Lamarre et al. 2001; Simmons, Clare et al. 2003). This susceptibility to systemic immunity was reversed by adoptive transfer of immune sera, suggesting that antibodies play a crucial role during infection (Simmons, Clare et al. 2003).

Studies have shown that infection of STEC and EPEC in humans leads to elevated humoral and cell mediated immune responses (Li, Frey et al. 2000; Tacket, Sztein et al. 2000). Although, *C. rodentium* infection can cause an inflammatory response with elevated cytokines that recruit neutrophils and macrophages, antibodies to A/E virulence factors can also inhibit bacterial attachment to the epithelial cells and reduce shedding (Potter, Klashinsky et al. 2004; La Ragione, Patel et al. 2006). Therefore, an antibody response could be an alternate to phagocytosis, as EPEC and STEC can inhibit this process during infection (Marches, Covarelli et al. 2008)

Passive immunization studies using colostrum have highlighted the importance of antibodies in limiting STEC infection. It was recently demonstrated that in a murine model the oral administration of immunoglobulin enriched bovine colostrum containing antibodies against numerous bacterial antigens was able to protect against challenge with STEC O157 (Funatogawa, Ide et al. 2002). Widiasih and colleagues have recently shown that passive immunity through colostrum can effectively transfer antibodies against STEC O26, O111 and O157 to newborn calves (Widiasih, Matsuda et al. 2004). Passive immunity was confirmed by the fact that antibody titers in sera collected from the newborn calves rapidly increased.

The relevance of antibody was also highlighted when mice were orally vaccinated with STEC antigens combined with a liposome vehicle resulting in elevated IgA and IgG responses in both serum and intestinal lavage fluid in mice (Tana, Watarai et al. 2003). The intestinal lavage fluid was then used in *in vitro* inhibition assays and shown to inhibit the attachment of STEC O157 to the intestinal cells. The overall importance of antibody in limiting a STEC infection in cattle was demonstrated using subcutaneous vaccination with Type III Secretion proteins. The production of IgG antibodies was induced and correlated with a reduction in the number of shedding animals, as well as the level of STEC shed in fecal samples (Potter, Klashinsky et al. 2004).

1.5 Treatment, control and prevention of STEC

1.5.1 Treatment of STEC

At this time, the most common treatment for STEC infections appears to be supportive care. The best plan for the prevention of HUS is to prevent the initial infection and spread of STEC (Tarr, Gordon et al. 2005). Several agents such as antimotility, narcotics and nonsteroidal medications should be discouraged. Antimotility pharmacological agents have been found to increase the probability of developing HUS and neurological effects (Cimolai, Morrison et al. 1992; Siegler and Oakes 2005). These agents can also cause seizures, as well as renal failure. In order to protect renal function, patients are put on rehydration with isotonic solutions (Ake, Jelacic et al. 2005). The use of peritoneal dialysis from the initial stages of infection to the point of recovery have shown to limit disease and mortality and should be encouraged. Rapid analysis of stool from suspected STEC infected patients should be completed by the diagnostic laboratory. This will alert physicians of the risk of HUS development allowing for immediate volume expansion (Ake, Jelacic et al. 2005).

The use of antibiotics among suspected STEC infected patients should be restricted. A study in 2000 reported a significant increase in the development of HUS in children who were treated with antibiotics, compared to children who were not (Wong, Jelacic et al. 2000). Antibiotics are believed to increase the susceptibility of HUS by destroying STEC, which causes the liberation of Stx or the induction of the bacteriophages where the toxin genes are located (Siegler and Oakes 2005). *In vitro* results confirmed that certain antibiotics, such as ciprofloxacin, cefiximine, tetracycline and co-trimoxazole, increased the production and release of Stx by 169% to 436% among five different STEC strains (Walterspiel, Ashkenazi et al. 1992). However, the antibiotic rifaximin has been recently shown to not increase the production of Stxs, or the bacteriophage lysis on 26 O157 and 31 non-O157 strains tested (Ochoa, Chen et al. 2007).

Several toxin-binding agents have been developed that could bind to the toxin during infection and reduce its uptake. A study showed that a polymer of the Stx Gb3 receptor, which demonstrated high affinity and inhibition to Stx1 and Stx2, was able to

protect mice against fatal challenge of STEC O157 (Watanabe, Matsuoka et al. 2004). The oral dose of the Gb3 polymer protected mice, even after the infection was established. The mice also demonstrated reduced brain damage, and a reduction of serum levels to Stx. Another synthetic molecule called Synsorb-Pk which resembles the Gb3 receptor, was tested in a group of 150 children, and was found to have no effect in preventing HUS compared to placebo group. It was believed that the reason why this molecule failed was because it was administered too late into the infection (MacConnachie and Todd 2004). A recombinant bacterium that expresses the Stx receptor Gb3 on its surface was recently tested, and appears to absorb and neutralize Stx toxin. Mice that were given three daily oral doses of live bacteria, or formaldehyde-killed bacteria, were completely protected against STEC challenge even 48 hours post-challenge (Paton, Rogers et al. 2001).

Several monoclonal antibodies have been developed that could be given to a patient to neutralize the Stx. The advantage of monoclonal antibodies is the protein-protein interaction and the productivity capabilities. Smith and colleagues have developed a monoclonal antibody that can neutralize the cytotoxic and lethal properties of Stx1 (Smith, Carvalho et al. 2006). This group has also developed a chimeric Stx1B/Stx2A toxoid protein, which can be protective when vaccinating mice, and could be used to develop monoclonal antibodies against both toxins (Smith, Teel et al. 2006). Another group has also demonstrated that monoclonal antibodies against Stx2 showed specificity and binding affinity to Stx2. This product could be used a potential prophylactic or therapeutic agent for patients with an STEC infection (Akiyoshi, Rich et al. 2005).

1.5.2 Control and prevention of animal infection

1.5.2.1 Management methods

A number of interventions can be used to limit the spread of STEC infections. Due to its asymptomatic properties in cattle, complete eradication in a farm environment is unlikely. However, several practices can be applied to reduce shedding and colonization of animals. The reduction in shedding should result in a decrease of

contaminated water sources such as rivers, ponds, and lakes that are regularly contaminated by pasture run-off or underground water (McGowan, Wickersham et al. 1989; McCarthy, Barrett et al. 2001; Neely, Bell et al. 2004). This decline would also reduce human infection, as humans commonly use these water sources for consumption and recreational activities. In addition, the reduction in shedding by asymptomatic ruminants could also reduce the contamination of meat and animal products as well as indirectly reduce the contamination of produce.

The practice of spreading manure as fertilizer for crop fields can also increase the shedding of STEC (Jiang, Morgan et al. 2002). The method of proper composting prior to spreading on fields, can eradicate STEC O157 from manure, and should be used as a common practice (Lung, Lin et al. 2001). Proper composting can apply to silage which is commonly fed to animals, and has been shown to be a source of STEC infections (Fenlon and Wilson 2000).

Water quality can play a critical role in the spread of STEC. In reality, if the water tests positive for STEC, the majority of the time the cattle in the region also test positive, demonstrating that water can be a vehicle for STEC (Davis, Cloud-Hansen et al. 2005). The daily cleaning of water troughs, and the treatment of water with chlorine, electrolyzed water and ozonation have been tested, and proven to reduce colonization (LeJeune, Besser et al. 2004). The water source used to fill water troughs and for use in daily farm maintenance should also be regularly tested for the contamination with STEC, since these sources are at risk for contamination and spread to new areas (Dorner, Anderson et al. 2007). The hygiene of feed also represents a candidate for the exposure reduction of STEC, since it plays a role in the introduction of the bacteria to cattle (Crump, Griffin et al. 2002). In European countries, strict feed hygiene controls have been established which have lowered the prevalence of *Salmonella*, another common zoonotic pathogen in cattle and swine (Boqvist, Hansson et al. 2003). Feed management has been suggested to reduce the spread STEC, since certain feeds such as cottonseed, corn silage, barley and beet pulp appear to increase prevalence (Berg, McAllister et al. 2004).

The housing of calves in the same unit, prior to weaning, can increase the shedding of STEC O157 since calves shed STEC O157 at higher volumes, and more

often than adults (Cray and Moon 1995). These calves can then act as vehicles for the spread of STEC, once they are divided and housed with adults. Therefore, the housing of calves separately, and the assurance that pens are kept clean by the removal of mud, and the accumulation of urine and fecal matter, can reduce the prevalence and spread of STEC (Smith, Blackford et al. 2001). The scraping of alleyways used by cattle also reduces the shedding of STEC, since the common practice of jet washing actually increases STEC shedding (Garber, Wells et al. 1999). In addition, hide washing with a mixture of 1.6% sodium hydroxide, 4% trisodium phosphate, 4% chlorofoam, or 4% phosphoric acid can reduce the amount of STEC O157 found on beef carcasses prior to processing (Bosilevac, Nou et al. 2005). The removal of super-shedders is also important since these animals shed STEC at higher rates then regular shedders within a feedlot or pasture (Matthews, McKendrick et al. 2006).

1.5.2.2 Biological methods

The use of probiotics to manipulate the normal flora of an animal and to add a competitive element against STEC O157 has been suggested as a possibility to reduce shedding. Probiotics have been used in the cattle industry for decades to improve the well-being of an animal. Researchers have also isolated a number of *E. coli* strains, capable of producing colicins, which inhibit colonization of other *E. coli,* and have shown promise in displacing and reducing shedding of STEC O157 in live cattle (Zhao, Tkalcic et al. 2003). *Lactobacillus acidophilus* has also been shown to reduce the amount of STEC O157 in up to 50% of tested animals, and is currently being used in US feedlots (Brashears, Galyean et al. 2003).

Antimicrobials administered through feed can be used to reduce the shedding of STEC O157. A large study involving 73 feedlots including 689 pens, reported that the addition of antibiotics in the feed significantly reduced fecal shedding compared to the control groups (Sargeant, Sanderson et al. 2004). Another drug which has recently been approved for use in feed is ractopamine. Ractopamine when administered to animals is responsible for dramatic muscle growth, but it is not considered a steroid or hormone, rather a compound known as a beta-agonist. Recently, it was discovered that the addition of ractopamine to feed significantly reduces the shedding of STEC O157 compared to the

control groups is a large feedlot trial (Edrington, Callaway et al. 2006). However, the use of these compounds has become controversial due to the increase of antibiotic resistance worldwide. Neomycin sulfate used in cattle, has demonstrated a decrease in fecal shedding of STEC O157 (Galland, Hyatt et al. 2001). However, it belongs to the family of aminoglycosides, which includes gentamycin and streptomycin, regularly used in humans.

Another controversial method is the use of bacteriophages to reduce the colonization of STEC O157. Bacteria are commonly lysed by bacteriophages found in the intestine of animals (Barrow, Lovell et al. 1998). Mouse experiments have shown promise, where the oral administration of a phage cocktail rapidly removed STEC O157 from the gastrointestinal tract of mice and from the fecal matter (Tanji, Shimada et al. 2005). A separate cocktail of bacteriophages isolated from STEC O157 was also tested as a control agent to eradicate STEC O157 spread. This cocktail contained 3 distinct phages, which reduced a culture of STEC O157 5-fold in 1 hour at 37ºC (O'Flynn, Ross et al. 2004). When this cocktail was tested in a beef meat trial, the cocktail completely eliminated the presence of the bacterium in seven of nine cases. Recently, a second cocktail of four bacteriophages was tested against human and bovine STEC O157 isolates. A total of 422 isolates were tested where the combination of all four bacteriophages were able to successfully lyse all isolates tested (Niu, Johnson et al. 2009).

Two separate groups investigated the potential of administrating bacteriophages prior to infection by STEC O157 in cattle. One study involved the administration of STEC O157 bacteriophages at the rectal junction where it is believed that STEC O157 also colonizes (Sheng, Knecht et al. 2006). In this study, the average counts of STEC were significantly lower than the control groups. However, even though the bacteriophages persisted, 80% of cattle tested still remained infected with STEC O157. The second study provided more promising evidence, where the delivery of bacteriophages in milk at day -7, -6, -1, 0 and 1 to young calves resulted in the complete eradication of STEC O157 shedding after a period of 8 days (Johnson, Gyles et al. 2008). These results imply that phage therapy may effectively control the shedding of STEC O157 under the proper circumstances.

Bacteriophage cocktails have also been used to investigate the reduction of contamination of vegetables, ground beef and hard surfaces by STEC O157 (Abuladze, Li et al. 2008). In an experimental contamination of hard surfaces, a phage cocktail containing three STEC O157 lytic phages was capable of reducing the number of bacterium by 97%. When the phage cocktail was tested in food products, such as ground beef, broccoli, spinach and tomatoes, similar results were observed, where the reduction of STEC O157 ranged from 100% in spinach to 94% in tomato samples. These results demonstrate that bacteriophage may play a role in reducing the contamination of fruits, vegetables, ground beef and surfaces by STEC O157. However, an important obstacle with bacteriophage treatment in either cattle or food products is acquiring Canadian Food Inspection Agency (CFIA) approval and public acceptance.

1.5.3 Control and prevention of human infection

In order to reduce human infection from direct contact with animals or other humans, proper hygiene practices should be applied. At slaughter houses, correct manufacturing procedures and good hygiene should be applied according to regulations implemented by CFIA to reduce contamination of meat products and carcasses. Schools and children visiting farms or petting zoos, must be supervised by adults to ensure that proper hand washing is enforced, particularly prior to food consumption (Caprioli, Morabito et al. 2005). Food hygiene must also be applied to prevent transmission of STEC. This involves the separation of raw and ready-to-eat products to ensure that no cross-contamination occurs. The proper cooking of meat, particularly hamburgers and other ground meat products, should be implemented in restaurants and house holds.

If an outbreak occurs, several precautions can be taken to ensure that secondary transmission does not occur (Seto, Soller et al. 2007). These include hand washing, reducing contact with individuals with diarrhea, avoiding contact with feces, staying home from work, or school, when having diarrhea during an outbreak, and thorough preparation of food prior to consumption. Individuals with prolonged diarrhea, especially hemorrhagic colitis, should consult a physician so that proper diagnosis can be reached and further complications can be avoided. In addition, if an STEC infection is discovered, the source of infection can be quickly identified to lessen the spread.

1.5.4 Vaccines

Vaccines are another preventative measure that can be taken to reduce the number of STEC infections and HUS cases. The use of Stxs as a vaccine component to induce a strong immune response has been tested by several groups. Wen and colleagues developed a plant-based oral vaccine, which expressed Stx2 and induced Stx2-specific mucosal IgA and Stx2-neutralizing serum IgG (Wen, Teel et al. 2006). When tested in mice, this vaccine was able to protect against challenge with STEC. Other toxoid based vaccines have also been tested. One candidate used an inactive form of Stx, where a couple of amino acids are mutated in the B or A subunit (Yamasaki 2002). A recent study has demonstrated that the intranasal vaccination of mice with a HIS-tagged StxB including a mutant heat-labile enterotoxin prevented toxemia by STEC (Tsuji, Shimizu et al. 2008).

However, other bacterial components have also been tested as vaccine targets. In a number of enteric pathogens such as *S. typhimurium, Shigallae* and *V. cholerae*, surface LPS have been shown to induce protective immunity (Gupta, Szu et al. 1992; Robbins, Chu et al. 1992; Konadu, Lin et al. 2000). O-specific LPS conjugates are designed to induce serum anti-conjugate-IgG antibodies which can inhibit the colonization of enteric pathogens (Konadu, Robbins et al. 1994; Robbins, Schneerson et al. 1995). The use of O-specific polysaccharide from STEC O157 combined with the exotoxin A of *Pseudomonas aeruginosa* resulted in antibody titers 20-fold higher then the control group and appeared to be safe in young children tested (Ahmed, Li et al. 2006). A separate group also using the O-specific polysaccharide from STEC O157 combined with the exoprotein A of *P. aeruginosa*, demonstrated how vaccination elicited a 4-fold increase in anti-LPS IgG levels within a week in 80% of adult volunteers (Konadu, Robbins et al. 1994). This anti-LPS IgG antibody was shown to have bactericidal properties. Other studies using detoxified LPS conjugated with a non-toxic version of the B subunit of Stx1 were also able to elicite high titers in mice (Konadu, Donohue-Rolfe et al. 1999). This serum was then used in *in vivo* studies and shown to have bactericidal properties against STEC O157.

Lipopolysaccharide was also incorporated in a cocktail liposomal complete-core LPS-based vaccine from Gram negative strains *E. coli* K-12, E. coli R1, *Pseudomonas*

aeruginosa, and *Bacteroides fragilis* (Bennett-Guerrero, McIntosh et al. 2000). Sera collected from vaccinated rabbits reacted against a number of Gram negative bacteria including *E. coli* serotypes O1, O4, O6, O8, O12, O15, O18, O75, O86, O157, and O111 and numerous other bacteria such as *P. aeruginosa*, *Klebsiella pneumoniae* serotype O1, O2ab, and O3, *B. fragilis*, and *Bacteroides vulgatus*.. However, a disadvantage with this method is the possibility of raising cross-protective serum against bacterial species part of the normal flora.

A number of antigen-delivery vehicles have been used to transport important antigens for the purpose of developing a protective immune response. One in particular is the use of monophosphoryl lipid A containing liposomes composed of almitoylphosphatidylcholine, dipalmitoylphosphatidylserine and cholesterol (1: 1: 2, molar ratio) (PS-liposome) which induces a significant systemic and mucosal immune response (Han, Watarai et al. 1997; Watarai, Han et al. 1998). This method has also been tested with STEC, where the oral vaccination of STEC antigens with the PS-liposome resulted in a significant increase in IgA and IgG responses in both serum and intestinal lavage fluid in mice (Tana, Watarai et al. 2003). Using Caco-2 cells in *in vitro* experiments, the IgA and IgG antibodies present in the intestinal lavages were shown to inhibit the adhesion of STEC O157 to the epithelial cells.

Another method applied for the protection of a host against STEC O157 was the use of the *Salmonella landau* strain as a vaccine component. This *Salmonella* strain is of particular interest because it naturally expresses the O-antigen of STEC O157 and is avirulent in mice when given in high doses (Bundle 1985; Conlan, KuoLee et al. 1999). Conlen and colleagues demonstrated that the vaccination of mice by gavage with *S. landau* resulted in high titers to both the strain and the O-antigen of STEC O157, which persisted for several months. Following challenge with STEC O157, the mice showed some evidence of protection from colonization. The down side to this method is that protection would be serotype specific due to the nature of the O-antigen. However, this study reiterates that a local immune response against STEC O157 can increase the host resistance.

The novel idea of vaccinating animals to protect humans has sparked plenty of interest. A number of vaccines have been tested using STEC O157 virulence factors. A

study demonstrated how the systemic vaccination of cattle with H7 flagellin reduced the colonization and delayed peak bacterial shedding (McNeilly, Naylor et al. 2008). This vaccination resulted in high levels of IgG in serum, and high levels of IgA in nasal secretion. Interestingly, detectable levels of both IgA and IgG were also found in rectal secretions. Dean-Nystrom and colleagues demonstrated that the vaccination of dams using intimin was capable of protecting against challenge with STEC O157 (Dean-Nystrom, Gansheroff et al. 2002). Other potential vaccines have been tested using intimin as a component. A group vaccinated rabbits with a STEC strain containing a non-functional intimin protein that protected against challenge (Agin, Zhu et al. 2005). Another study has demonstrated that a plant-based vaccine, including the carboxy-terminal cell binding domain of intimin, given orally or intraperitoneolly, was proficient in reducing fecal shedding in mice challenged with STEC O157 (Judge, Mason et al. 2004). Although intimin appears to be a potential vaccine component, a cross-protective vaccine based on this protein would be challenging, as over 16 different variations have been identified throughout STEC serotypes (Garrido, Blanco et al. 2006).

In 2004 Potter *et al*, reported that the vaccination of cattle using secreted proteins of STEC O157 significantly reduced the numbers of bacteria shed in feces, the numbers of animals that shed, and the duration of shedding (Potter, Klashinsky et al. 2004). This vaccine has been recently tested in large scale feedlots and the results have been promising. A large feedlot study involving 20,556 cattle in 19 different feedlots demonstrated how a two-dose vaccination reduced the prevalence of fecal shedding and the probability of environmental transmission of STEC O157 (Smith, Moxley et al. 2008). Another large feedlot trial using the same two-dose vaccination system vaccine, showed that vaccination of the majority of cattle within a pen, demonstrated significant herd immunity following challenge with STEC O157 (Peterson, Klopfenstein et al. 2007).

2.0 HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

That an *E. coli* O157:H7 vaccine containing T3SPs and other components will be capable of protecting cattle against a broad range of non-O157 STEC serotypes.

2.2 Overall goals and rationale

Our hypothesis is based on results that vaccination with STEC O157:H7 T3SPs is protective against colonization of cattle with STEC O157:H7. The majority of non-O157 STEC serotypes produce A/E lesions, with the assistance of a T3SS that secretes effectors required for the colonization of cattle. These serotypes possess the LEE pathogenicity island, including all 41 ORFs, where many express proteins shown to play a conserved role in pathogenesis. We believe that vaccination with T3SPs from STEC O157:H7 will protect against a broad range of non-O157 STEC serotypes. We also believe that a T3SP supernatant-based vaccine or a cocktail of recombinant effector-based vaccine will be capable of inducing a humoral response which can reduce the number of bacteria shed in feces, the number of animals that shed the bacterium, as well as the duration of shedding.

1. To test the cross reactivity of T3SPs of serotypes O26:H11, O103:H2, O111:NM and O157:H7.
2. To develop a cross-protective vaccine against serotypes O26:H11, O103:H2, O111:NM and O157:H7.

2.3 Objectives

1. To characterize secreted proteins found in STEC serotypes O26:H11, O103:H2 and O111:NM.
2. Construction of a chimeric Tir protein containing numerous epitopes from non-O157 STEC serotypes.
3. To measure the cross-reactivaty and cross-protective properties of a recombinant secreted protein vaccine.

3.0 GENERAL MATERIALS AND METHODS

3.1 Bacterial strains and growth conditions

Bacterial strains used in this study included *E. coli* EDL933 (O157:H7) (Tarr, Neill et al. 1989) obtained from a HUS patient and kindly provided by Dr. B Brett Finlay (Michael Smith Laboratories Biochemistry & Molecular Biology Microbiology & Immunology, Vancouver, BC, Canada) (Table 3.1). All non-O157 STEC serotypes such as CL101 (O111:NM), CL9(O26:H11), and N01-2454 (O103:H2) (Karmali, Mascarenhas et al. 2003) were kindly provided by Dr. Mohamed Karmali (Laboratory for Foodborne Zoonoses Public Health Agency of Canada, Guelph, ON, Canada). Strains were stored at -70^{o}C in 30% glycerol and were grown in Luria-Bertani (LB) agar and LB broth (DIFCO, Becton-dickinson, Sparks, MD, USA) with the appropriate antibiotics at 37^{o}C with shaking.

3.2 Preparation of competent cells

Bacterial *E. coli* strains were grown in 1 L of SOB Medium (contains 20 g/L of Bacto Tryptone, 5 g/L of Yeast Extract, 0.58 g/L of NaCl and 19 g/L of KCl followed by 4.07 g/L of $MgCl_2$ $6H_2O$ after autoclaving) at 37^{o}C with shaking to an absorbance at OD_{600} of 0.6 (approximately 5.5 hours). The cultures were centrifuged at 4,000 RPM for 10 minutes at 4 ^{o}C. The cell pellets were then resuspended in 333.3 mL of RF1 solution (contains 12 g/L of RbCl, 9.9 g/L of $MnCl_2$ $4H_2O$, 2.94 g/L of CH_3COOK, 1.5 g/L of $CaCl_2$ and 150 g/L of glycerol, pH adjusted to 5.8 with 12.01 g/L of CH_3CO_2H), placed on ice for a period of 15 minutes followed by centrifugation at 8,000 RPM for 10 minutes at 4 ^{o}C. The pellets were then resuspended in 80 mL of RF2 solution (contains 2.09 g/L of 3-(N- morpholino) propanesulfonic acid (MOPS), 1.2 g/L of RbCl, 11 g/L of $CaCl_2$ $2H_2O$ and 150 g/L of glycerol, pH adjusted to 6.8 with NaOH) and once again placed on ice for 15 minutes. The volume was then distributed into 1 mL aliquots, flash frozen and stored at -70 ^{o}C.

Table 3.1 List of bacterial strains and plasmids used.

Strain	Description	Reference
STEC strains		
E. coli EDL933 O157:H7	Human clinical isolate	(Potter, Klashinsky et al. 2004)
CL101 O111:NM	Human clinical isolate	(Karmali, Mascarenhas et al. 2003)
CL9 O26:H11	Human clinical isolate	(Karmali, Mascarenhas et al. 2003)
N01-2454 O103:H2	Human clinical isolate	(Karmali, Mascarenhas et al. 2003)
***E. coli* strains**		
DH5	Used for the expression of Leukotoxin fused STEC gene	Laboratory stock
JM109	Used for the expression of histidine-tagged STEC genes	Laboratory stock
M15	Used for the expression of histidine-tagged STEC genes	Laboratory stock
BL21	Used for the expression of GST-tagged STEC genes	Laboratory stock
Plasmids		
pQE-30	Prokaryotic cell expression vector	Qiagen
pGEX-5X-1	Prokaryotic cell expression vector	Pharmacia
pAA352-Tir	Prokaryotic cell expression vector	Laboratory stock

3.3 Transformation of competent cells

Competent cells were removed from -70 ^{o}C and thawed at room temperature. A volume of 200 μL of cells was transferred to a 15 mL pre-chilled plastic centrifuge tube. The quantity of DNA material to be transformed was added to the centrifuge tube, swirled and placed on ice for 30 minutes. Tubes are then heat shocked at 42 ^{o}C for 90 seconds without agitation, and placed on ice for 2 minutes. The tubes were then removed from the ice and 800 μL of SOC medium [contains 3.6 g/L of glucose, 4.07 g/L of $MgCl_2$ $6H_2O$, 4.33 of $MgSO_4$ $7H_2O$ per L of SOB medium (BIO 101 Inc, Vista, CA, USA)] was added. Samples were incubated at 37 ^{o}C with shaking for 60 minutes, followed by centrifugation for 10 minutes at 2,000 RPM. The supernatant was discarded and the pellet re-suspended in the remaining supernatant and spread on LB agar with appropriate antibiotic. Plates are incubated at 37 ^{o}C overnight.

3.4 Production of Type III secreted proteins.

Type III secreted proteins from all STEC serotypes were prepared essentially as described (Li, Frey et al. 2000). Briefly, overnight cultures of bacteria were diluted 100 fold in M9 minimal medium (Sigma-Aldrich, St. Louis, MO, USA) (Adams 1959) supplemented with 4g/L of glucose, 50 g/L of Casamino acids, $NaHCO_2$, 6.24 g/L of $MgSO_4$ and 4.51 g/L of $KHCO_3$. The cultures were then incubated without shaking at 37^{o}C in a 5% CO_2 environment to an absorbance at OD_{600} of 0.6-0.8. Cells were sedimented by centrifugation and the supernatant concentrated by precipitation with 10% Trichloroacetic acid. Samples were incubated with Trichloroacetic acid (111 μL per 1 mL of sample) for a period of 60 minutes on ice. Samples are then centrifuged at 15,000 RPM for 60 minutes and the supernatant solution discarded. The cell pellets were then resuspended in an appropriate buffer.

3.5 SDS-PAGE and Western Blot analysis.

Protein samples where mixed with equal volumes of 2X protein loading dye [100 mM Tris HCl, 4% sodium dodecyl sulphate (SDS), 0.2 % bromophenol blue, 20% glycerol and 10% mercaptoethanol]. Samples are then heated for 5 min in a boiling water bath and loaded into the proper lane of a sodium dodecyl sulphate polyacrylamide gel

(SDS-PAGE). Proteins were separated using a power source (Model 250/2.5 BioRad) and visualized following staining with Coomassie Brilliant Blue (20% MeOH and 10% Glacial Acetic acid and 0.5 g/L of Coomassie Brilliant Blue) for a period of 30 minutes and destained (20% MeOH and 10% Glacial Acetic acid) for 2 hours with regular buffer exchange (Laemmli 1970). For Western blot analysis, proteins were transferred to nitrocellulose membranes by electroblotting as recommended by the manufacturer (BioRad Laboratories). Briefly, the protein separation gels were soaked in transfer buffer (3 g/L tris, 1.41 g/L glycine, 20% MeOH) and placed on a nitrocellulose membrane (BioRad) with a piece of pre-cut Whatman filter paper on either side. This combination was placed in a power supply chamber (PowerPac200, BioRad) with chilled transfer buffer. The buffer was then kept cold with an ice filled plastic reservoir. The proteins were transferred to the nitrocellulose membrane at a current of 0.3 mA for a period of 45 minutes. After transfer, the nitrocellulose membrane was placed in block solution (8.8 g/L of NaCl, 0.2 g/L of KCl, 3 g/L of Tris base and 500 µL of Tween-20, pH adjusted to 7.4) (TBS-T) with 3 % skim milk for 1 hour at room temperature. After incubation, the membrane was washed three times with TBS-T and incubated with primary antibody concentration for 1 hour at room temperature. After the incubation, the membrane was again washed three times with TBS-T and incubated with secondary antibody (1/2000) for 1 hour at room temperature. After the final antibody incubation, the membrane was washed once with TBS-T and once with alkaline (AP) buffer (12.11 g/L of Tris base, 5.84 g/L of NaCl, 1.04 g/L of $MgCl_2$ $6H_2O$, pH adjusted to 9.5). The membranes were then incubated for 15 minutes with AP buffer and developed using nitroblue tetrazolium (NBT) salt (Sigma) and 5-bromo-4-chro-3-indolyl phosphate (BCIP) (Sigma) (De Jong, Van Kessel-van Vark et al. 1985).

3.6 Enzyme-Linked Immunosorbent Assay

Serological responses were measured using an enzyme-linked immunosorbent assay (ELISA) as described (Potter, Klashinsky et al. 2004). Antigen of choice was diluted to a concentration of 1 g/L in sodium carbonate buffer (1.5 g/L of $NaCO_4$, 2.93 g/L $NaHCO_4$, pH adjusted to 9.6), and 100 µL plated per well of a 96-well plate (Immulon 2 HB, Thermo) and incubated overnight at 4 ^oC. The next morning, the plates

were washed six times with distilled water and blocked for 1 hour at room temperature with TBS-T with 1% skim milk. Serum of choice was then serially diluted in TBS-T with 1% skim milk in four-fold dilutions starting at a dilution of 1:40, and incubated for 2 hours at room temperature (100 μL per well). After the incubation period, the plates were washed six times with distilled water, and incubated for 1 hour with the secondary antibody in TBS-T with 1% skim milk (100 μL per well at a dilution of 1/2000). Plates were then washed and incubated to desired time with 100 μL of 1 g/L freshly prepared para-nitrophenyl phosphate (PNPP) (Sigma) in PNPP substrate buffer [10 mL/L of Diethanolamine (Sigma) and 47.61 g/L of $MgCl_2$, with pH adjusted to 9.8 with HCl]. The absorbance was read on a microtiter plate reader at 405 nm with reference at 490 nm, while the serum titer was calculated by the intersection of least-square regression of A_{405} versus logarithm of dilution.

3.7 Expression and purification of HIS-tagged proteins

An overnight LB culture was inoculated at 1:100 into fresh LB + ampicillin (100 μg/mL). Culture was grown at 37ºC with shaking to an absorbance at OD_{600} of 0.6, and induced for 3 hrs with 0.1mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were pelleted and HIS-tagged proteins were purified with Ni-NTA slurry (Qiagen) under denaturing conditions using the protocol from QIAexpressionist (Qiagen). Briefly, pellet is suspended in 5 mL of Buffer A (13.8 g/L of NaH_2PO_4 H_2O, 1.2 g/L of tris base, 573 g/L of guanidine hydrochloride, with pH adjusted to 8.0 using NaOH) or B (13.8 g/L of NaH_2PO_4 H_2O, 1.2 g/L of tris base, 480.5 g/L of urea, with pH adjusted to 8.0 using NaOH) and incubated for 1 hour. Cells are centrifuged at 10,000 *g* for 20 minutes at room temperature. Supernatant is mixed with 1 mL of 50% Ni-NTA slurry (Qiagen) per 4 mL lysate and placed on a rotary shaker for 1 hour at room temperature. The lysate-resin mixture is put through a column and washed twice with 4 mL buffer C (13.8 g/L of NaH_2PO_4 H_2O, 1.2 g/L of tris base, 480.5 g/L of urea, with pH adjusted to 6.3 using HCl). The HIS-tagged protein is then eluted four times with 0.5 mL of buffer D (13.8 g/L of NaH_2PO_4 H_2O, 1.2 g/L of tris base, 480.5 g/L of urea, with pH adjusted to 5.9 using HCl) followed by four times with buffer E (13.8 g/L of NaH_2PO_4 H_2O, 1.2 g/L of Tris

base, 480.5 g/L of urea, with pH adjusted to 4.5 using HCl). The purity of proteins was evaluated by SDS-PAGE using Coomassie Brilliant Blue (Laemmli 1970).

4.0 CROSS REACTIVITY OF ENTEROHEMORRHAGIC ESCHERICHIA COLI O157:H7-SPECIFIC SERA WITH NON-O157 SEROTYPES

4.1 Introduction

Shiga toxin-producing *Escherichia coli* are significant zoonotic pathogens of humans that are capable of causing severe gastrointestinal disease that can lead to a number of sequelae, including Hemorrhagic Colitis, HUS (Van Dyck, Proesmans et al. 1988; Tuttle, Gomez et al. 1999; Karmali, Petric et al. 2004) and TTP (Morrison, Tyrrell et al. 1986). The association between HUS and STEC infection has been well documented over the past two decades (Kulkarni, Goldwater et al. 2002) and is due to the production of one or more Shiga toxins which may be produced by the organism, alone or in combination (Dean-Nystrom, Gansheroff et al. 2002; Friedrich, Bielaszewska et al. 2002; Karmali, Petric et al. 2004). Hemolytic-uremic syndrome is the most common cause of acute renal failure in children.

The most prominent STEC serotype associated with disease in North America is O157:H7 (DeVinney, Stein et al. 1999; Elder, Keen et al. 2000). The CDC has estimated that O157:H7 strains cause approximately 73,000 illnesses and 60 deaths per year in the USA, while non-O157 STEC strains cause an additional 37,000 cases (Mead, Slutsker et al. 1999). Non-O157 STEC serotypes, commonly associated with human disease, include O26:H11, 0103:H2, O111:NM and O113:H21 (Paton, Ratcliff et al. 1996; Karmali, Petric et al. 2004). In many countries, including Australia, Chile and Argentina, non-O157 STEC serotypes are associated with the majority of HUS cases (Karmali, Petric et al. 2004). The incidence of HUS in Argentina has been reported to be 5-10 times higher than in North America (Lopez, Diaz et al. 1989) and a recent Italian study demonstrated an increase in infections caused by non-O157 serotypes over the past five years (Tozzi, Caprioli et al. 2003). Taken together, these observations suggest that STEC strains of serotypes other than O157:H7 are an increasing source of disease on a global basis.

Colonization of the host by STEC strains is mediated through the action of a T3SS which delivers effector molecules, including the translocated intimin receptor Tir, into host cells (DeVinney, Stein et al. 1999). The organism subsequently binds to Tir via the outer membrane receptor, intimin (DeVinney, Stein et al. 1999; Frankel, Phillips et al.

2001). Many of the genes coding for the structural components of the T3SS, as well as effector proteins, are located on the locus of enterocyte effacement (LEE) pathogenicity island (McDaniel, Jarvis et al. 1995; Perna, Mayhew et al. 1998). However, effector molecules involved in colonization and disease are also encoded by genes located at other sites on the chromosome (Karmali, Mascarenhas et al. 2003). While Type III secreted proteins are required for virulence (Donnenberg, Kaper et al. 1997), they have also been shown to be targets of the immune system in humans (Li, Frey et al. 2000). These proteins are also required for colonization of cattle, the primary reservoir of *E. coli* O157:H7. Certain non-O157 serotypes also produce a T3SS and have been associated with cattle (Frankel, Phillips et al. 1998).

A number of methods are being developed to control levels of *E. coli* O157:H7 in cattle, including the use of probiotics (Zhao, Doyle et al. 1998), bacteriophages (Kudva, Jelacic et al. 1999), treatment of drinking water (Rice, Clark et al. 1999) and vaccination (Potter, Klashinsky et al. 2004). Vaccination with T3SPs has been shown to reduce shedding of the organism under both experimental and field conditions (Potter, Klashinsky et al. 2004) and antibodies against structural components of the Type III secretion system (EspA) as well as effector molecules (Tir) appear to be associated with decreased colonization. In order to be broadly cross-protective, a vaccine will also need to block colonization by non-O157 serotypes. Thus, the objective of this study was to determine the degree of serological relatedness between T3SPs of serogroups O26, O103, O111 and O157.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth conditions

Bacterial strains used in this study included *E. coli* EDL933 (O157:H7) (Tarr, Neill et al. 1989), CL101 (O111:NM), CL9 (O26:H11), and N01-2454 (O103:H2) (Karmali, Mascarenhas et al. 2003). Strains were stored at -70^{o}C in 30% glycerol and were grown on LB agar and LB broth at 37^{o}C. All non-O157 STEC strains were kindly provided by Dr. M. Karmali (Laboratory for Foodborne Zoonoses, Guelph, Ontario). For visualization by immunofluorescence microscopy, strains of all serotypes were transformed with plasmid pNR78 which expresses the gene for the green fluorescent protein (Quantum Biotechnologies) under the control of the GroEL promoter. Recombinant Tir and EspA were produced in *E. coli* K12 as described (Li, Frey et al. 2000).

4.2.2 Sequence analysis.

Extraction of genomic DNA from *E. coli* strain N01-2454 was carried out as described (Sambrook 1989) and the *tir* gene was amplified using primers based upon the O26:H11 sequence. The sequence of the forward primer (Tir_F) and reverse primer (Tir_R) were (5'-GCCTATTGGTAATCTTGGCCACAATCCC-3') and (5'-TAAACGAAACGTGCGGGTCCCGGCGTTG-3'), respectively. Sequences of the PCR product were determined using overlapping primers on a CEQ200 model sequencer as recommended by the manufacturer (Beckman-Coulter).

4.2.3 Production of Type III secreted proteins

Type III secreted proteins from all STEC serotypes were prepared essentially as described (Li, Frey et al. 2000). Briefly, overnight cultures of bacteria were diluted 100 fold in M9 minimal medium (DeVinney, Stein et al. 1999) supplemented with 0.4% glucose, 0.1% Casamino acids, 44 mM $NaHCO_2$, 8 mM $MgSO_4$ and 45 mM $KHCO_3$. Cultures were then incubated without shaking at 37^{o}C in a 5% CO_2 environment to an OD_{600} of 0.6-0.8. Cells were pelleted by centrifugation and the supernatant concentrated by precipitation with 10% Trichloroacetic acid dissolved in water.

4.2.4 SDS-PAGE and Western blot analysis

Proteins were separated by SDS-PAGE and were visualized following staining with Coomassie Brilliant Blue (Laemmli 1970). Proteins were transferred to nitrocellulose membranes by electroblotting as recommended by the manufacturer (BioRad Laboratories) and as described (Section 3.5). Western blot analysis was then carried out using anti-Tir, anti-EspA and anti-STEC T3SPs antibodies as described (Laemmli 1970; Li, Frey et al. 2000). Bovine polyclonal antibodies against serotype O157:H7 T3SPs were raised as described (Potter, Klashinsky et al. 2004). Anti-$EspA_{O157}$ and anti-Tir_{O157} monoclonal antibodies were acquired from Bioniche Life Sciences (Belleville, Canada).

4.2.5 Real-Time reverse transcriptase PCR

Total RNA from the strains described was prepared using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Contaminating DNA was removed by treatment with Dnase 1 (Qiagen) in RNeasy mini columns (Qiagen). The RNA concentration and integrity was determined by capillary electrophoresis on a Bioanalyzer (Agilent Technologies). Oligonucleotides used for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) were designed with Beacon designer V2.13 (PREMIER Biosoft International) and were synthesized by Sigma Laboratories The sequence of the forward primer (Tir_{rtF}) and reverse primer (Tir_{rtR}) were (5'-TCAGACCTCAACCTCAACTTC-3') and (5'-AATACCCGTCGCCGCCAACCCTATC-3'), respectively. Complementary DNA was synthesized using Superscript III Reverse transcriptase (Invitrogen) following priming with the oligonucleotides described above. Quantitative RT-PCR and data analyses were performed as described (Fortin, Mulchandani et al. 2001). Briefly, The qRT-PCR reaction was performed in 96 well plates (BioRad) with the iCycler^{TM} iQ real-time PCR detection system (BioRad) for 45 cycles of 95ºC for 15sec, 55ºC for 30 sec and 72ºC for 30sec after holding for 2 min at 50C and 95ºC, respectively. The qRT-PCR was completed using Platinum SYBR green qPCR SuperMix-UDG (Invitrogen). Triplicate qRT-PCR reactions were performed for each STEC serotype. After real-time data

acquisition, the cycle threshold (C_T) value was calculated by determining the point at which fluoresce exceeded an arbitrary threshold signal (10-fold higher than base line).

4.2.6 Construction of Tir deletions for the identification of epitope recognized by anti-Tir monoclonal antibody

Three different sized fragments of STEC O157 EDL933 *tir* gene from the N-terminus [fragment A = 559 base pairs, fragment B= 1118 base pairs and fragment C= 1677 base pairs (full sized *tir* gene)] were initially selected for the identification of the epitope recognized by an anti-Tir monoclonal antibody. Primers used for the construction of fragments are described in Table 4.1. A copy of each fragment was amplified by PCR and cloned into the pUEX1 plasmid (GenBank Accession L08867), which was digested with *BamH*I and *Pst*I for fragment A and B and *Bgl*II and *Pst*I for fragment C, followed by ligation. Plasmids were transformed and bacteria were grown as described (Howell and Hargreaves 1988).

Eight other deletions in the *tir* gene were constructed from the N-terminus of the Tir protein. These deletions were named N-terminus 100, N-terminus 200, N-terminus 300, N-terminus 400, N-terminus 220, N-terminus 240, N-terminus 260 and N-terminus 280 based on the number of deleted base pairs from the 5' end of the *tir* gene. Primers used for the construction of deletions are described in Table 4.1. All fragments were amplified by PCR, digested with *Bgl*II and *Pst*I and ligated into the pUEX1 plasmid (GenBank Accession L08867). Plasmids were then transformed and bacteria were grown as described (Howell and Hargreaves 1988).

4.2.7 Expression of *tir* deletions

Cloned fragments of *tir* were expressed in plasmid pUEX1 as described (Howell and Hargreaves 1988). Briefly, 2 mL cultures were grown overnight at 30ºC. A dilution at 1/50 from the overnight culture was made in 10 mL of LB broth and grown with shaking to an absorbance at OD_{600} of 0.6. The bacterial culture was then split into two 5 mL aliquots and one was placed at 30ºC as the control sample while the other was placed at 42 ºC to allow the expression of *tir* deletions proteins by removing the temperature

Table 4.1. Oligonucleotide primers used for the construction of STEC *tir* deletions. Nucleotide sequence is from 5' to 3'. F= forward primer; R= reverse primer. Primers were used for PCR for the construction of fragments. Fragments were digested with the appropriate restriction enzymes and cloned into the pUEX1 plasmid (GenBank Accession L08867), followed by ligation. Values listed on primer names represent the number of deleted nucleotides from the *tir* gene.

Primers	Restriction sites
Fragment A -F	
CGCGGATCCCCTATTGGTAACCTTGGTCATAATCC	BamHI
Fragment A -R	
GCTACTGCAGTTACTTTGGGCTCTAACAGCTCCAG	PstI
Fragment B-F	
CGCGGATCCCCTATTGGTAACCTTGGTCATAATCC	BamHI
Fragment B-R	
GCTACTGCAGTTACCACCAAGAATCAATGCGCCACTAAG	PstI
Fragment C-F	
GGAAGATCTCCTATTGGTAACCTTGGTCATAATCC	BglII
Fragment C-R	
GCTACTGCAGTTAGACGAAACGATGGGATC	PstI
N-terminus 100-F	
GGAAGATCTCTCATTAACTCTACGGGGCCGTTGGG	BglII
N-terminus 100-R	
GCTACTGCAGTTAGACGAAACGATGGGATC	PstI
N-terminus 200-F	
GGAAGATCTGGACTTCCTGTAAATCCGATG	BglII
N-terminus 200-R	
GCTACTGCAGTTAGACGAAACGATGGGATC	PstI
N-terminus 300-F	
GGAAGATCTCAGATTGGCTCTTCGGTATTTCG	BglII
N-terminus 300-R	
GCTACTGCAGTTAGACGAAACGATGGGATC	PstI
N-terminus 400-F	
GGAAGATCTATCAAGAGTACGCTCGCTTG	BglII
N-terminus 400-R	
GCTACTGCAGTTAGACGAAACGATGGGATC	PstI
N-terminus 220-F	
GGAAGATCTCGCCTGGCGGCGTCTGAGATAAC	BglII
N-terminus 220-R	
GCTACTGCAGTTAGACGAAACGATGGGATC	PstI
N-terminus240-F	
GGAAGATCTATAACACTGAATGATGGATTTG	BglII
N-terminus 240-R	
GCTACTGCAGTTAGACGAAACGATGGGATC	PstI
N-terminus 260-F	
GGAAGATCTGAAGTTCTTCATGATCATG	BglII
N-terminus 260-R	
GCTACTGCAGTTAGACGAAACGATGGGATC	PstI
N-terminus 280-F	
GGAAGATCTGGTCCGCTCGATACTCTTAAC	BglII
N-terminus 280-R	
GCTACTGCAGTTAGACGAAACGATGGGATC	PstI

repression of the λ promoter. A volume of 1.5 mL of culture was centrifuged for 5min at 20,000 g to pellet the cells. The pellet was resuspended in 120 µL of 2X loading buffer with 10% mercaptoethanol and the sample was boiled for 5 minutes. A total of 3 µL was used on a SDS-PAGE gel for Western blot analysis.

4.2.8 Vaccination with Type III secreted proteins

Vaccines containing T3SPs from the strains described above were prepared by formulating 100 µg of total protein with the adjuvant VSA3 as described (Potter, Klashinsky et al. 2004). Groups of 8 six-month old calves were vaccinated twice with T3SPs from one of four STEC strains with 21 days between each immunization. The placebo group received an identical formulation without antigen (Potter, Klashinsky et al. 2004). Serum samples were collected at the time of each immunization as well as two weeks following the second vaccination. The Tir, EspA and T3SP-specific serological responses were measured using an ELISA as described (Potter, Klashinsky et al. 2004), using T3SPs antigen prepared from homologous and heterologous serotypes. Differences were considered significant at a P-value < 0.05 (Wilcoxon Rank Sum Test) using sera collected on day 21.

4.2.9 Adherence Inhibition assays

The adherence of STEC O157:H7 and non-O157 serotypes of *E. coli* to HEp-2 cells was examined by a modification of the assay of Leverton *et al* (Leverton and Kaper 2005). Bacterial strains were subcultured from an overnight culture in LB broth into pre-warmed DMEM at a 1/50 dilution and this culture was then incubated without shaking for a period for 2 hours at 37ºC in 5% CO_2. Eight-well chamber slides were seeded with 2 x 10^5 HEp-2 cells and incubated overnight at 37ºC in 5% CO_2. At the time of infection, DMEM was removed from chambers and replaced with a combination of 225 µl of fresh DMEM, 25 µL (O157:H7 O103:H2, O111:NM) or 50 µl (O26:H11) of bacterial culture and 12.5 µL of homologous or heterologous pre- and post immunization bovine antiserum per well. Infections were allowed to proceed at 37ºC in 5% CO_2 for 3 hours (O157:H7), 3.5 hours (O26:H11) or 4 hours (O103:H2, O111:NM). Slides were washed 6 times with phosphate-buffered saline and air dried. A coverslip was added and slides

were visualized using immunofluorescence microscopy. Homologous and heterologous sera used in the Adherence Inhibition assays were collected on day 21 after vaccination. A total of eight chambers were prepared per sample and four grids from each were selected randomly and bacteria per cell counted. Each data point represents the average of these values from a single experiment.

4.2.10 Preparation of STEC O157:H7 acetone powder and its absorption against rabbit polyclonal antibody

A culture of STEC O157:H7 in LB was grown overnight with shaking at 37ºC. Bacterial cells from a 350 mL culture were pelleted, the supernatant removed and pellet resuspended in approximately 4 mL of saline. The resuspended culture was transferred to ice for 5 minutes. A volume of 16 mL of cold Acetone (Merck) was added to the bacterial cell suspension, mixed vigorously and incubated at 0ºC for 30 minutes with occasional mixing. The solution was then centrifuged for 10 minutes at 10, 000 x g and the supernatant was discarded. The pellet was resuspended in cold Acetone (Merck), mixed vigorously and incubated at 0ºC for 10 minutes. The precipitate was collected by centrifugation for 10 minutes at 10,000 x g. The pellet was then transferred to a clean piece of 3 MM filter paper (Whatman) and the precipitate was spread and allowed to air-dry 1 hour at room temperature. Any large pieces which did not break into fine powder were removed.

A total of 0.1 gram of acetone powder was added to 1 mL of rabbit sera containing polyclonal antibody raised against STEC O157 T3SPs. The rabbit sera was raised by immunizing female New Zealand white rabbits subcutaneously with one millilitre quantity containing 50 μg of STEC O157 T3SPs, and 30% of Emulsigen® - D (MVP laboratories). The animals received two boosts, three weeks apart before being euthanized. The serum sample containing the acetone powder was placed on a nutator (Clay Adams brand) to mix for 30 minutes at 4 ºC. After the 30 minutes, the sample was centrifuged for 10 minutes at 10, 000 x g and the supernatant was collected. The supernatant was subsequently used in the adherence inhibition assay.

4.3 Results

4.3.1 Cross reactivity of Tir and EspA specific antibodies

To measure the degree of cross-reactivity of immune responses against T3SPs of serotypes O157:H7, O26:H11, O103:H2 and O111:NM, we focused initially on measuring responses against EspA, a protein involved in the translocation of effector proteins, including Tir. Both monoclonal and polyclonal antibodies specific for serotype O157:H7 proteins were used. The $EspA_{O157}$ monoclonal antibody was capable of recognizing the EspA protein produced by serotypes O26:H11 and O111:NM (Figure 4.1a), but it did not react with EspA of serotype O103:H2. An identical result was obtained using $EspA_{O157}$-specific polyclonal antibodies (Figure 4.2a).

In contrast, a Tir_{O157}-specific monoclonal antibody did not react against Tir from any of the heterologous serotypes. Tir_{O157}-specific polyclonal antibodies did react with Tir from all serotypes (Figure 4.2b) albeit to a lesser extent than against the serotype O157:H7 proteins. The least reactive T3SPs were those produced by serotype O103:H2, similar to the result obtained using the anti-EspA monoclonal antibody (see above). Although the same amount of total protein was used from each serotype, the concentration of individual components could have differed due to differences in transcriptional regulation. In order to determine if this was the case, we carried out qRT-PCR analysis of *tir* transcripts and the results showed similar levels of RNA from each serotype (Figure 4.3). The STEC O26 cycle threshold mean was 16.2, the STEC O103 cycle threshold mean was 18.7 and the STEC O111 cycle threshold mean was 22.6.

4.3.2 Identification of the epitope recognized by an anti-Tir monoclonal antibody

A number of deletions in the Tir protein were constructed to identify the location of the Tir epitope recognized by the anti-Tir monoclonal antibody supplied by Bioniche Life Sciences. Initially, three constructs were expressed which divided the Tir protein into three different sized fragments [fragment A = 559 base pairs, fragment B= 1118 base pairs and fragment C= 1677 base pairs (full sized *tir* gene)] (Figure 4.4). After the completion of a Western blot analysis, it was observed that all three fragments reacted

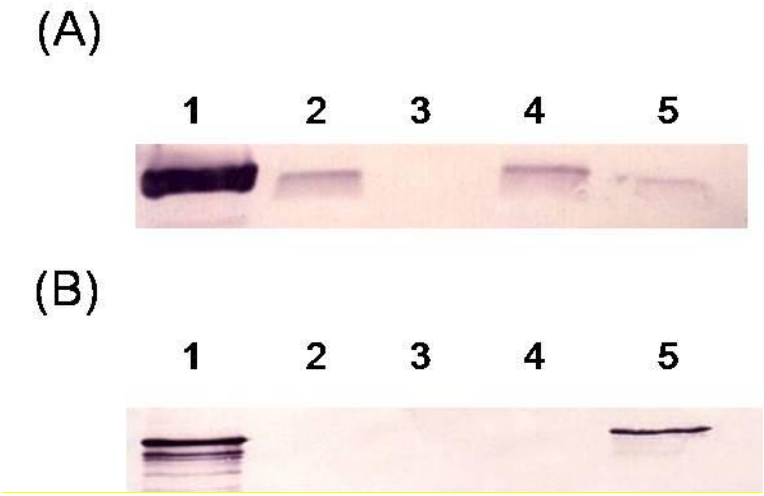


Figure 4.1 Western blot analysis using anti-Tir$_{O157}$ and anti-EspA $_{O157}$ monoclonal antibodies against non-O157 STEC serotypes. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-EspA$_{O157}$ (Panel A) and anti-Tir$_{O157}$ (Panel B) monoclonal antibodies Lane 1,purified Tir or EspA: lane 2, O26:H11 T3SPs: lane 3, O103:H2 T3SPs: lane 4, O111:NM T3SPs: lane 5, O157:H7 T3SPs.

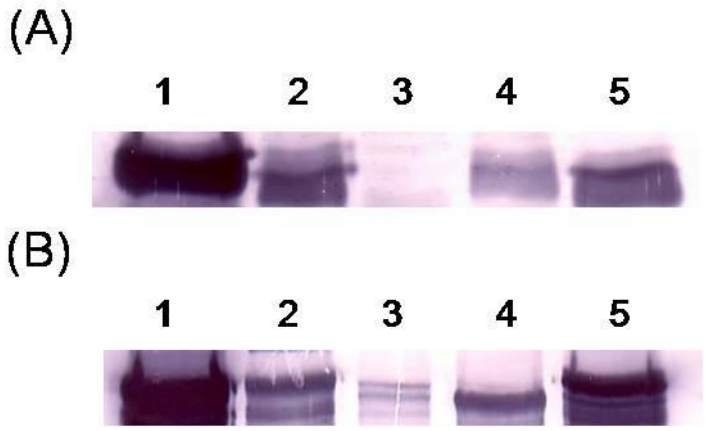
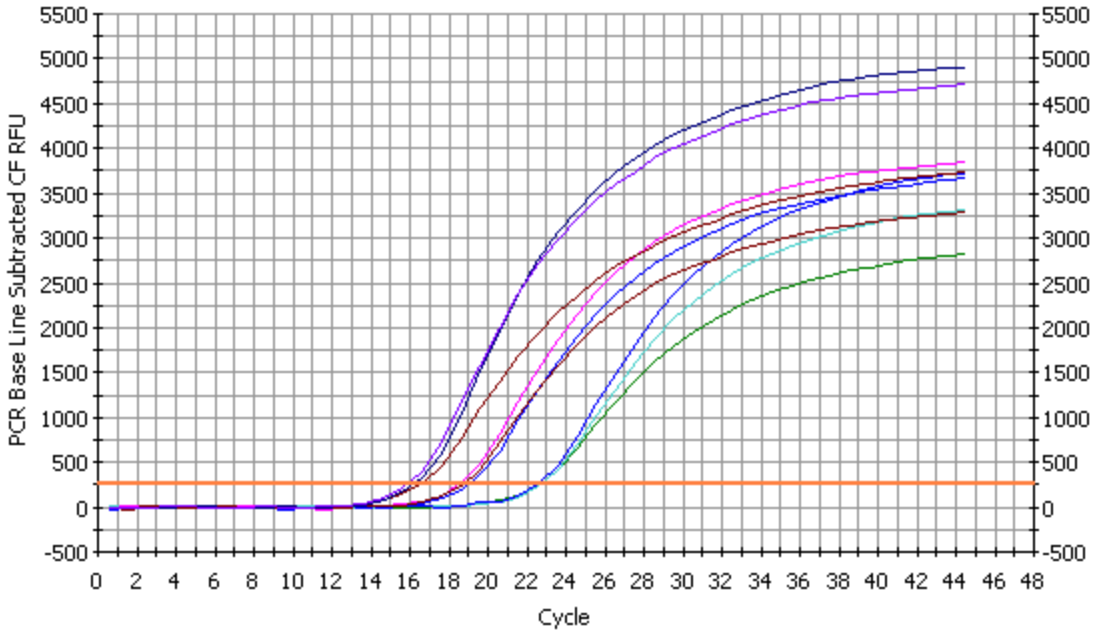
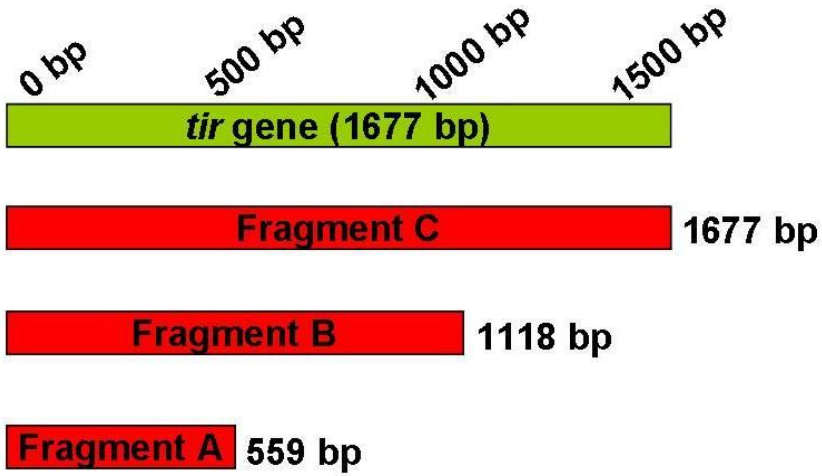


Figure 4.2 Western blot analysis using anti-Tir$_{O157}$ and anti-EspA $_{O157}$ polyclonal antibodies against non-O157 STEC serotypes. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-EspA $_{O157}$ (Panel A) and anti-Tir $_{O157}$ (Panel B) polyclonal antibodies Lane 1, purified Tir or EspA: lane 2, O26:H11 T3SPs: lane 3, O103:H2 T3SPs: lane 4, O111:NM T3SPs: lane5, O157:H7 T3SPs.



STEC Serotypes	Ct (Cycle Threshold)
O26:H11	15.9
O26:H11	16.2
O26:H11	16.5
O103:H2	19
O103:H2	18.5
O103:H2	18.7
O157:H7	22.7
O157:H7	22.7
O157:H7	22.5

Figure 4.3 qRT-PCR results demonstrating cycle threshold for the transcription of the *tir* gene by three STEC serotypes (O26:H11, O103:H2, O157:H7). Quantitative RT-PCR and data analyses were performed as described (Fortin, Mulchandani et al. 2001). Briefly, The qRT-PCR was performed in 96 well plates (BioRad) with iCycler^{TM} iQ real-time PCR detection system (BioRad) for 45 cycles of 95ºC for 15sec, 55ºC for 30sec and 72ºC for 30sec after holding for 2 min at 50ºC and 95ºC respectively. qRT-PCR was completed using Platinum SYBR green qPCR SuperMix-UDG (Invitrogen). Triplicate qRT-PCR reactions were performed for each STEC serotype. After real-time data acquisition, the cycle threshold (C_T) value was calculated by determining the point at which fluoresnce exceeded an arbitrary threshold signal (10-fold higher than the base line).


0 bp
500 bp
1000 bp
1500 bp
tir gene (1677 bp)
Fragment C
1677 bp
Fragment B
1118 bp
Fragment A
559 bp

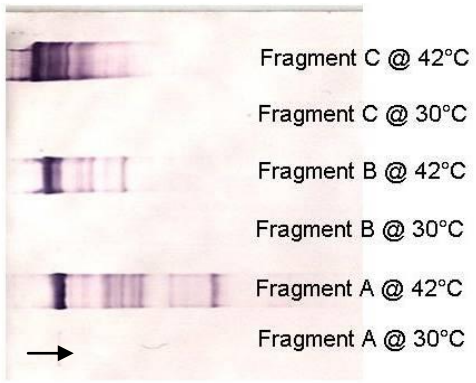

Fragment C @ 42°C
Fragment C @ 30°C
Fragment B @ 42°C
Fragment B @ 30°C
Fragment A @ 42°C
Fragment A @ 30°C

Figure 4.4. Identification of the epitope recognized by the anti-Tir O157:H7 monoclonal antibody. Three different sized fragments of STEC O157 EDL933 *tir* gene from the N-terminus [fragment A = 559 base pairs, fragment B= 1118 base pairs and fragment C= 1677 base pairs (full sized *tir* gene)] were initially selected for the identification of the epitope recognized by the Tir monoclonal antibody. All three fragments reacted with the monoclonal antibody. The lanes at 30ºC represent the control samples which are expected to be negative due to repression of the promoter. 42 ºC samples allow for the expression of *tir* deletions by removing the temperature repression of the λ promoter. These results demonstrate that the epitope recognized by the Tir monoclonal antibody is present on Fragment A. The arrow on the Western blot represents migrating path of proteins.

with the anti-Tir monoclonal antibody. These results demonstrate that sequence coding for the epitope is located on the first 559 nucleotides (fragment A) of the *tir* gene from the 5' to 3'.

Based on the above results, we constructed four other *tir* gene deletions where the first 500 nucleotides were deleted 100 nucleotides at a time. These deletions were named N-terminus 100, N-terminus 200, N-terminus 300 and N-terminus 400 based on the number of deleted base pairs from the 5' end of the gene. After constructs were expressed and the products analyzed by Western blotting, both the N-terminus 100 and N-terminus 200 deletions reacted against the anti-Tir monoclonal antibody, while no reactivity was observed with the other two deletions (Figure 4.5). These results demonstrate that the starting point of the epitope recognized by the anti-Tir monoclonal antibody is between the first 200 and 300 nucleotides of the *tir* gene from the 5' end. This area is identified on Figure 4.5.

To further map the position of the epitope, four *tir* gene deletions between the nucleotides 200-300 from the 5' end of the *tir* gene were constructed. Each one of these deletions eliminated 20 nucleotides from this region of the *tir* gene. These deletions were named N-terminus 200, N-terminus 240, N-terminus 260 and N-terminus 280 based on the number of deleted base pairs from the 5' end of the *tir* gene. After constructs were expressed and the products used in Western blot analysis, it was observed that 3 out of 4 deletions reacted with the anti-Tir monoclonal antibody, only the N-terminus 280 fragment failed to react (Figure 4.6). These results demonstrate that the starting point of the epitope is located between nucleotides 260-280 of the *tir* gene. This area is identified on Figure 4.6.

An amino acid sequence alignment was completed on the Tir protein from STEC serotype O157, O26, O103 and O111 (Figure 4.7). The entire sequence alignment between all serotypes revealed a significant amount of variation, which also appears to be evenly distributed. In the region recognized by the monoclonal antibody of the suspected epitope is located, a large amount of variability was observed (Figure 4.7B).

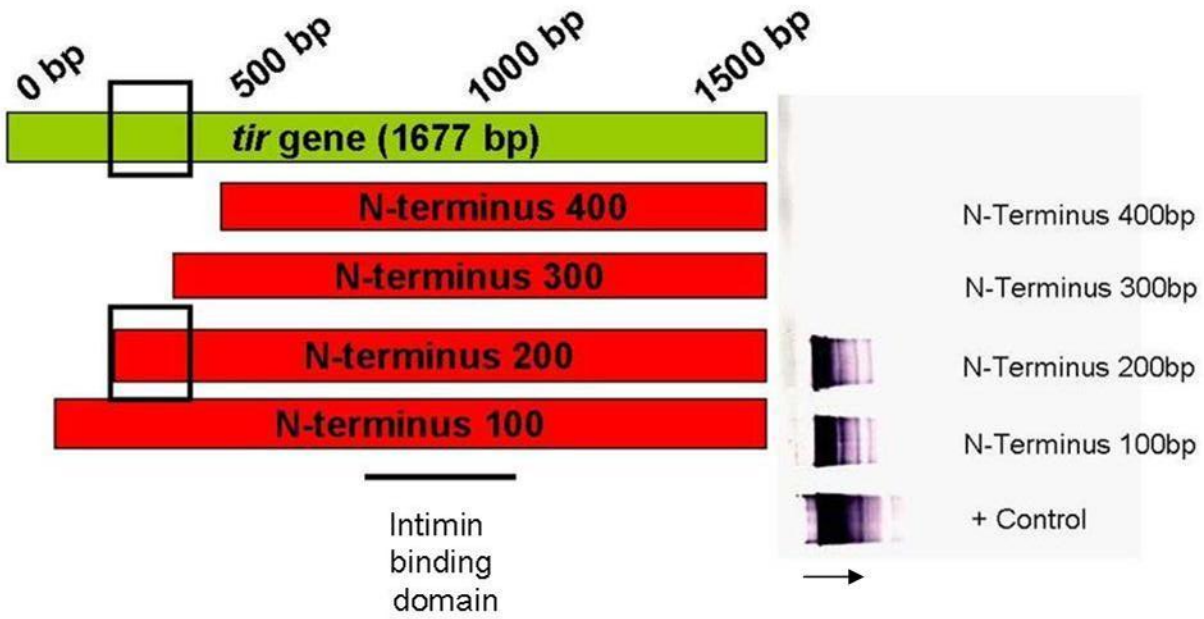

0 bp
500 bp
1000 bp
1500 bp
tir gene (1677 bp)
N-terminus 400
N-terminus 300
N-terminus 200
N-terminus 100
Intimin binding domain
N-Terminus 400bp
N-Terminus 300bp
N-Terminus 200bp
N-Terminus 100bp
+ Control

Figure 4.5. Identification of the N-terminus starting point of the epitope recognized by the anti-Tir O157:H7 monoclonal antibody within fragment A. Four Tir deletions were constructed based on the reactive fragment A from the N-terminus of the *tir* gene. These deletions were named N-terminus 100, N-terminus 200, N-terminus 300 and N-terminus 400 based on the number of deleted base pairs from the 5' end. Two of the four deletions reacted with the monoclonal antibody. Although both control samples at 30ºC and expression samples at 42 ºC were expressed, only the 42 ºC samples were analysed by Western blot. These results demonstrate the epitope recognized by the Tir monoclonal antibody starts within 200 bases of the N-terminus of the *tir* gene. The deletion where the epitope begins is highlighted by the black box. The arrow on the Western blot represents migrating path of proteins.

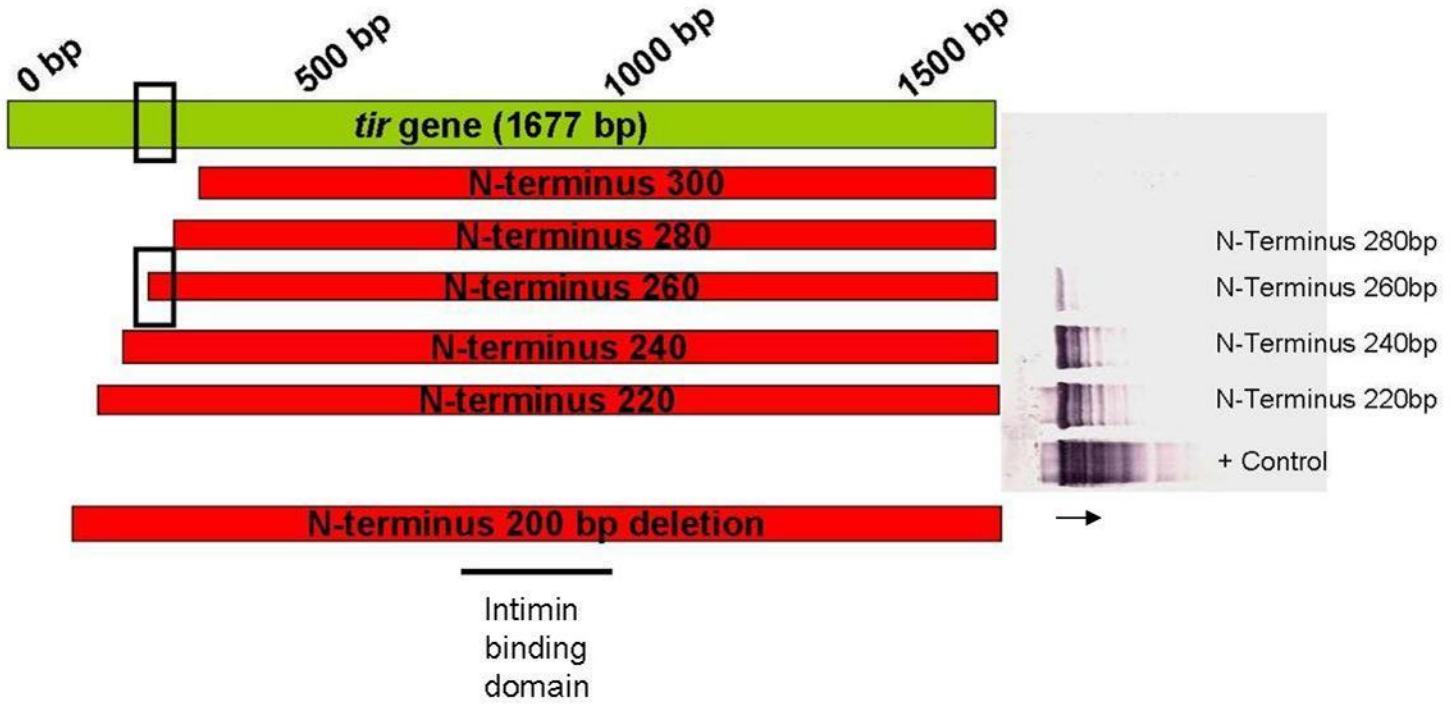

0 bp
500 bp
1000 bp
1500 bp
tir gene (1677 bp)
N-terminus 300
N-terminus 280
N-terminus 260
N-terminus 240
N-terminus 220
N-terminus 200 bp deletion
Intimin binding domain
N-Terminus 280bp
N-Terminus 260bp
N-Terminus 240bp
N-Terminus 220bp
+ Control

Figure 4.6. Identification of the N-terminus starting point of the epitope recognized by the anti-Tir O157:H7 monoclonal antibody within the N-terminus 200 deletion. Four Tir deletions were constructed based on the last reactive Tir deletion from Figure 4.5. (N-terminus 200). These deletions were named N-terminus 220, N-terminus 240, N-terminus 260 and N-terminus 280 based on the number of deleted base pairs from the 5' end. Three of the four deletions reacted with the monoclonal antibody. Although both control samples at 30ºC and expression samples at 42 ºC were expressed, only the 42 ºC samples were analyzed by Western blot. These results demonstrate the epitope recognized by the Tir monoclonal antibody starts within 260 bases of the N-terminus of the *tir* gene. The deletion where the epitope begins is highlighted by the black box. The arrow on the Western blot represents migrating path of proteins.

A)

```
O157    1 mpignlghnpnvnnsippapplpsqtdga--gg-rgqlinstgplgsralftpvrnsmadsgdnrasdvpglpvnpmrlaa--seitlndgfevlhdhgpldtlnrqigssvfrvetqedgkhiavgqrn
O26     1 mpignlghnpnvralippapplpsqtdga--ggarnqlinsngpmgsrllftpirnsvadaadsrasdipglptnplrfaa--sevslhgalevlhdkggldtlnsaigsslfrvetrddgshvaigqkn
O103    1 mpignlghnpnvralippapplpsqtdga--ggarnqlinsngpmgsrllftpirnsvadaadsrasdipglptnplrfaa--sevslhgalevlhdkggldtlnsaigsslfrvetrddgshvaigqkn
O111    1 mpignlgnnvnsnnlippapplpsqtdgasrgg-agqlinstgalgsrllfsplrnsiadsvdsr--dipglpvhpsrlatatseicllggfevlhdkgpldtlnkqigasafrieqqsdgsyaaigekn

O157  126 gvetsvvlsdqeyarlqsidpegkdkfvftggrggaghamvtvasditearqrilellepkgtgeskgag---eskgvgelresnsgaent--tetqtststsslrsdpklwlalgtvatgliglaatgi
O26   127 glettvvlseqefsslqsldpegknkfvftggrggaghamvtvasdiaearqriidklepkdtketkepg-----------dpnsgegki--ieihtststsslradpklwlslgtiaagligmaatgi
O103  127 glettvvlsdqefsslqsldpegknkfvftggrggaghamvtvasdiaearqriidklepkdtketkepg-----------dpnsgegki--ieihtststsslradpklwlslgtiaagligmaatgi
O111  128 gvevsvilnsqelqslqaidiedkgrfvftggrggghsmvtpasdiaearakilakldpnnhggsqarnvdtrsvgvg----sasgmddsvvsetrtsstassvrsdpkfwvsigaiaaglaglaatgi

O157  251 vqalaltpepdspttdpdaaasatetatrdqltkeafqnpdnqkvnidelgnaipsgvlkddvvanieeqakaageeakqqaiennaqaqkkydeqqakrqeelkvssgagyglsgalilgggigvavt
O26   243 aqavaltpepddpittdpdaaantaeaaakdqltkeafqnpdnqkvnidengnaipsgelkddvvaqiaeqakaageqarqeaiesnsqaqqkydeqhakreqemslssgvgygisgalilgggigagvt
O103  243 aqavaltpepddptttdpdtaastaeaatkdrltqeafqdpdkqkvnidengnaipsgeliddvvaqiaeqakaageqarqeaiesnsqaqkkydeqhakreqemslssgvgygisgalilgggigagvt
O111  254 tqalaltpepddptttdpeqaasaaesatrdqltqeafknpenqkvsideignsipsgelkddvvakieeqakeageaarqqavesnaqaqqrydtqyarrqeelelssgigyslssalivgggigagvt

O157  381 aalhrknqpveqttttttttttsartvenkpannptaqgnvdtpgsedtmesrrssmastsstff-dtssigtvqnpyadvktslhds--qvptsnsntsvqnmgntdsvvystiqhpprdttdngarl
O26   373 aalhrknqpaeqtittrt--------vvdnqptnnasaqgntdtsgpeespasrrnsnaslasngs-dtsstgtvenpyadvgmprnds--lariseepiydevaadpn---ysviqhfsgnspvtg-rl
O103  373 aalhrknqpaeqtittrt--------vvdnqptnnasaqgntdtsgpeespasrrnsnaslasngs-dtsstgtvenpyadvgmprndslaripeepiydevaadpn-----ysviqhfsgnspvtg-rl
O111  384 talhrrnqpaeqttttttht------vvqqqtggntpaqggtdairaedtslnrrdsqrstasthwsdtss--avvnpyaevgearnss----parqaeehiydevaadp-nysviqnfsgnnqvtg-rl

O157  508 lgnpsagiqstyarlal-sgglrhdmggltggsnsavntsnnppapgshrfv
O26   488 vgtpgqgiqstyallas-sgglrlgmggltgggesavstanaaptpgparfv
O103  488 vgtpgqgiqstyallas-sgglrlgmggltgggesavstanasptpgparfv
O111  500 mgtpgqgiqstyailtnnsaglrlgmggltgsggsavntanaaptpgpgrfv
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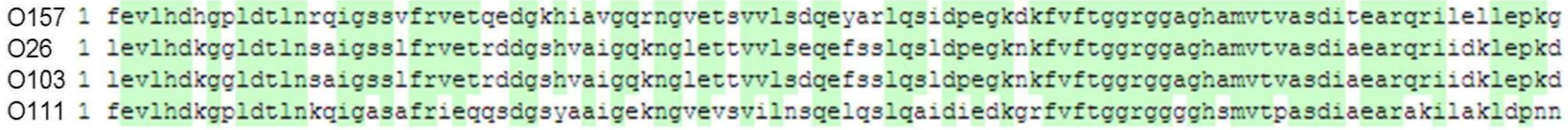
B)


O157 1 fevlhdhgpldtlnrqigssvfrvetqedgkhiavgqrngvetsvvlsdqeyarlqsidpegkdkfvftggrggaghamvtvasditearqrilellepkg
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O103 1 levlhdkggldtlnsaigsslfrvetrddgshvaigqknglettvvlsdqefsslqsldpegknkfvftggrggaghamvtvasdiaearqriidklepkd
O111 1 fevlhdkgpldtlnkqigasafrieqqsdgsyaaigekngvevsvilnsqelqslqaidiedkgrfvftggrggggghsmvtpasdiaearakilakldpnn

Figure 4.7. Sequence alignment of Tir protein from STEC O157 and non-O157 serotypes. A) Whole Tir sequence alignment. Amino acid sequence was aligned for serotype STEC O157, O26, O103 and O111 B) Amino acid region (101 AA) representing the location of the epitope recognized by the anti-Tir monoclonal antibody. The letters in white correspond to the variable amino acids while the green correspond to the homologous amino acids. (-) represent missing amino acids for that specific serotype. Based on the predicted sequence of the Tir protein for STEC serotype O157 (GenBank NC_002655), serotype O103 (GenBank AJ303141), serotype O26 (GenBank AJ277443) and serotype O111 (GenBank AF025311).

4.3.3 Immunization of cattle with Type III secreted proteins of various STEC serotypes

Since the sera used in the experiment described was directed against only O157 proteins, cattle were immunized with T3SPs from all four serotypes in order to determine individual cross-reactivities. All groups of cattle demonstrated a significant serological response following a single immunization (Figure 4.8), a trend which continued after a second dose of vaccine was administered. The increase in antibody levels ranges from 23-fold following a single immunization to 69-fold following the boost. When ELISA titers were calculated against heterologous T3SPs (Figure 4.9) a different pattern was observed. Animals vaccinated with the O26:H11 proteins did not have a significant titer against the heterologous antigens while those animals vaccinated with T3SPs from the other three serotypes showed varying degrees of cross reactivity, with the group which received the O111:NM vaccine formulation showing the highest degree of cross reactivity. However, all were significantly lower than the response against the homologous serotype. The Tir-specific and EspA-specific responses were also measured using antigen produced from serotype O157:H7 (Figure 4.10). No significant cross reactivity was seen for either antigen with the exception of the group that received the O157:H7 vaccine formulation. Taken together, these results suggest that there is significant antigenic variability in T3SPs produced by these four serotypes.

4.3.4 Adherence Inhibition of STEC serotypes

Sera collected from cattle vaccinated with T3SPs were also used in a functional inhibition assay in order to study its capacity to inhibit adherence of STEC serotypes to HEp-2 cells. Hyperimmune sera were able to strongly inhibit the adherence of homologous STEC serotypes when compared to the pre-immune sera (Figure 4.11). However, when heterologous sera were tested, the level of inhibition was decreased in all groups. The greatest degree of inhibition of attachment by heterologous sera was observed with O157:H7 strain (Figure 4.11d), while attachment of serotype O111:NM was inhibited the least (Figure 4.11c). Overall the capacity of sera to inhibit attachment of heterologous strains was significantly lower than for the homologous strain. These

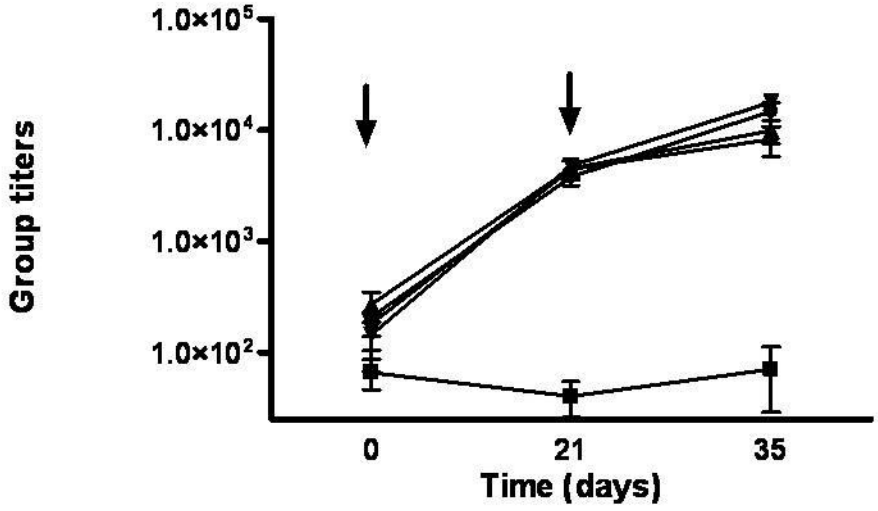


Figure 4.8 Serological responses following vaccination of cattle with Type III secreted proteins. The response against T3SPs of the homologous serotype for each vaccinated group was measured by ELISA as described above. (■) Placebo, (▲) O26, (▼) O103, (♦) O111, (●) O157. Arrows represent vaccinations given at day 0 and day 21.

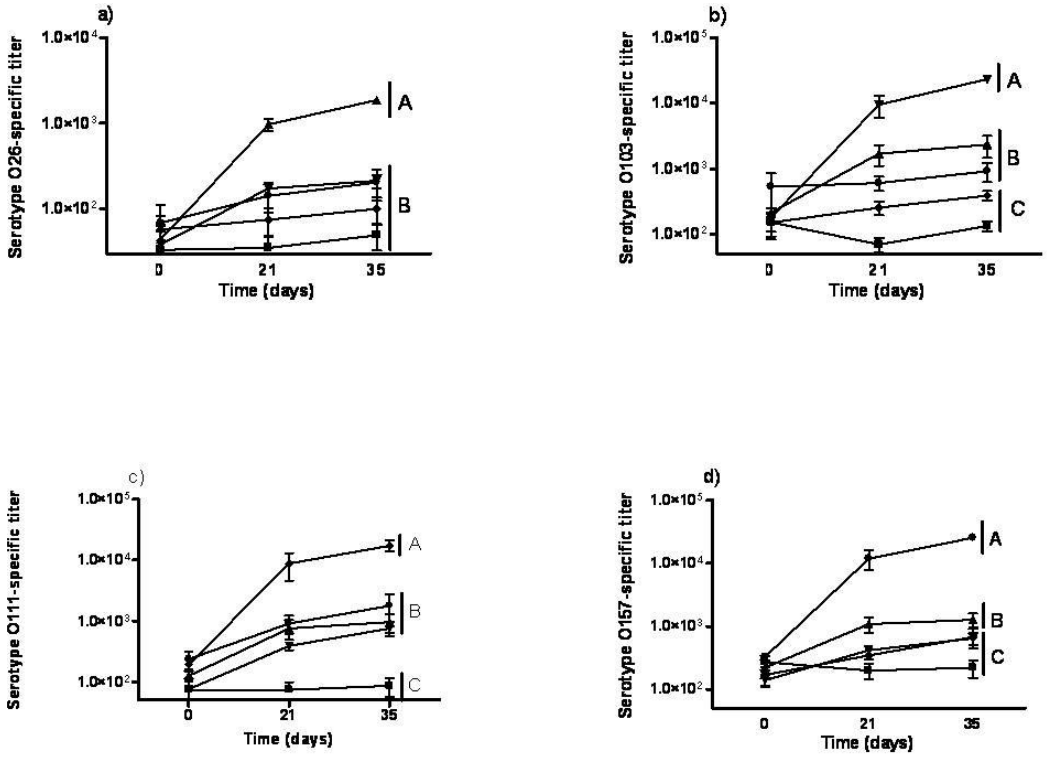


Figure 4.9 Cross reactivity of sera against Type III secreted proteins from heterologous serotypes. Panel a, responses against O26 T3SPs; Panel b, O103 T3SPs; Panel c, O111 T3SPs; Panel d, O157 T3SPs; (■) Placebo, (▲) O26, (▼) O103, (♦) O111, (●) O157. Responses were grouped statistically [A, B, C ; Wilcoxon Rank Sum Test ($p < 0.05$)] using sera collected on day 21.

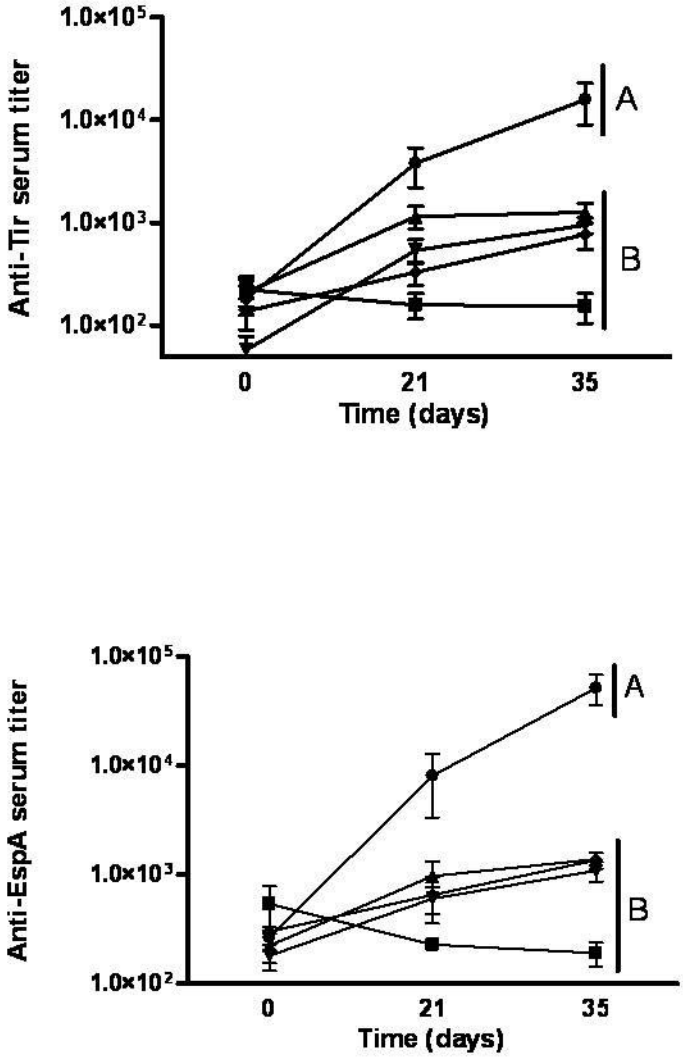


Figure 4.10 Reactivity of sera collected from cattle vaccinated with Type III secreted proteins from serotypes O26:H11, 0103:H2, O111:NM, and O157:H7 against Tir_{O157} and $EspA_{O157}$. (■) Placebo, (▲) O26, (▼) O103, (♦) O111, (●) O157. Responses were grouped statistically [A, B; Wilcoxon Rank Sum Test ($p < 0.05$)] using sera collected on day 21.

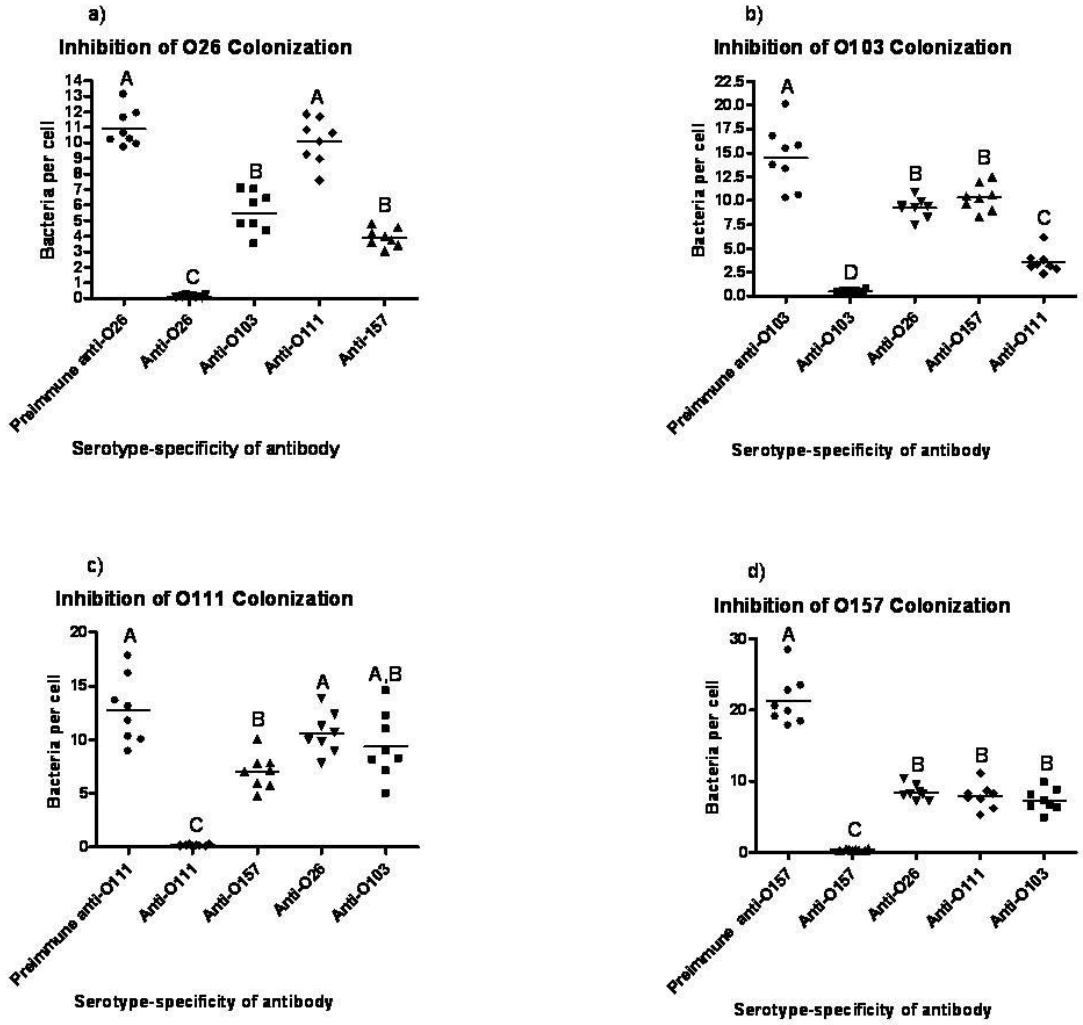


Figure 4.11 Inhibition of colonization of STEC serotypes by homologous and heterologous sera raised against T3SPs. Panel a, inhibition of O26 colonization; Panel b, inhibition of 0103 colonization; Panel c, inhibition of O111 colonization; Panel d, inhibition of O157 colonization; (●) Preimmune sera, (▲) anti-O157, (▼) anti-O26 , (♦) anti-O111, (■) anti-O103. [A, B, C ; Wilcoxon Rank Sum Test ($p < 0.05$)] using sera collected on day 21.

results further suggest significant antigenic variability within T3SPs of the serotypes tested.

4.3.5 Adherence Inhibition of STEC O157 using absorbed and unabsorbed sera

In order to determine if the adherence inhibition observed was due to T3SPs or surface proteins and/or LPS, rabbit serum raised against STEC O157 T3SPs was absorbed with acetone powder prepared from STEC O157 whole cells. Both the absorbed and unabsorbed rabbit serum were still capable of inhibiting the adherence of STEC O157 when compared to the preimmune serum (Figure 4.12). In addition, both the absorbed and unabsorbed rabbit serum were used in a Western blot analysis to confirm that a reduction in antibody raised against other possible proteins aside from T3SPs was removed (Figure 4.13). When the cell lysate was used, a clear difference can be seen with both seras tested, where the unabsorbed serum reacts with components of the lysate while the absorbed serum does not. Both the absorbed and unabsorbed sera reacted with STEC O157 T3SPs in the Western blot. The absorbed serum produced bands that were much sharper than those produced by the unabsorbed serum. Overall, these results demonstrate that the adherence inhibition observed is mainly due to serum raised against T3SPs.

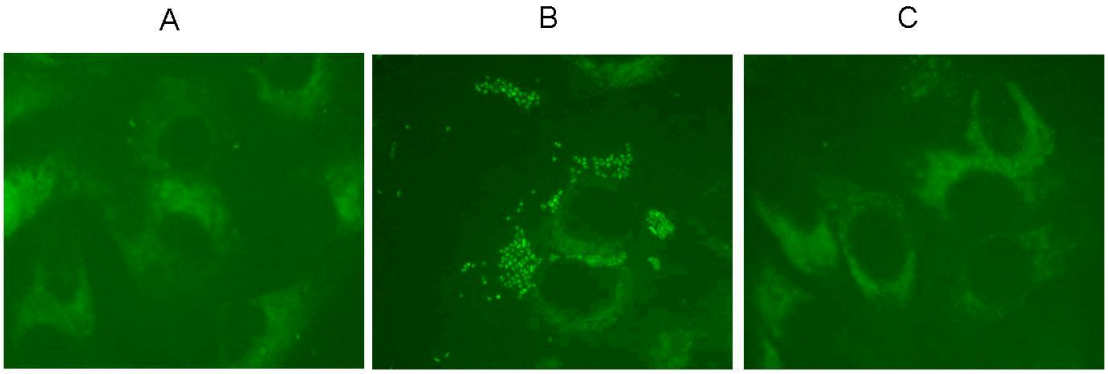


Figure 4.12 Inhibition of colonization of STEC O157 by absorbed and unabsorbed rabbit serum raised against STEC O157 T3SPs. Absorbed serum was treated with acetone powder made from STEC O157. Panel A, inhibition of O157:H7 colonization with unabsorbed rabbit serum; Panel B, inhibition of O157:H7 colonization with preimmune serum; Panel C, inhibition of O157:H7 colonization with absorbed rabbit serum.

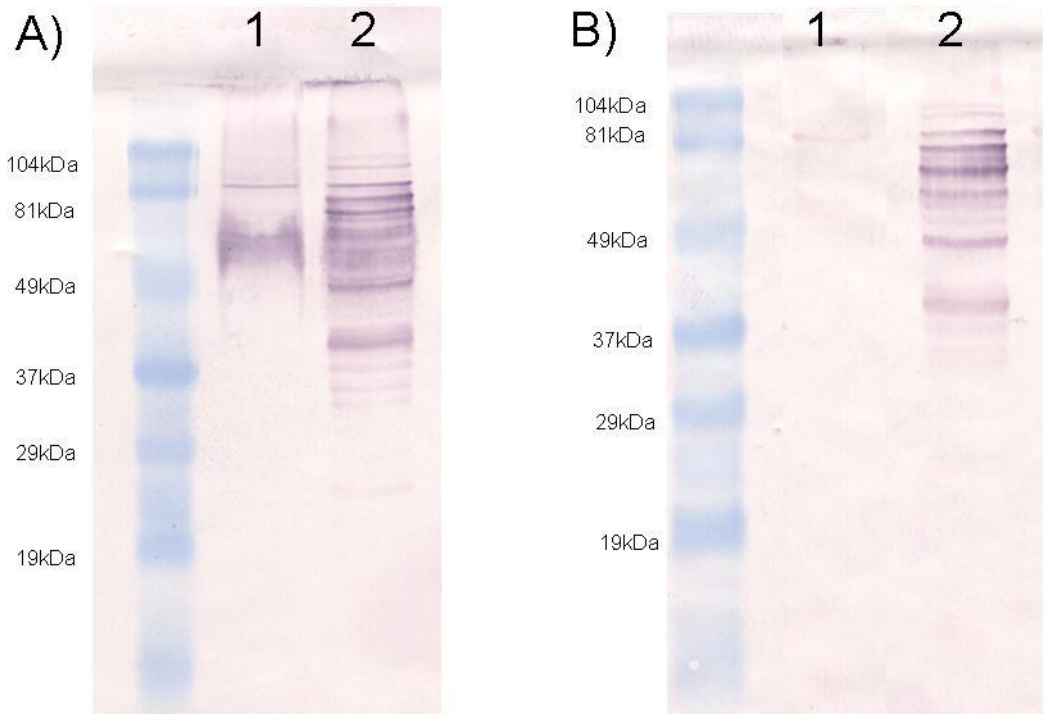


Figure 4.13 Western blots using absorbed and unabsorbed rabbit serum raised against STEC O157 T3SPs. Absorbed serum was treated with acetone powder made from STEC O157. A) Western blot using unabsorbed sera B) Western blot using absorbed sera. Lane 1 = cell lysate from STEC O157. Lane 2 = T3SPs from STEC O157.

4.4 Discussion

The objective of this study was to determine the degree of immunological cross reactivity between the serotype O157:H7 T3SPs and those of other STEC serotypes. We chose to monitor responses against Tir and EspA since they represented both a secreted effector and a member of the translocation apparatus, respectively, and have been shown to induce immune responses in humans and animals following natural infection and vaccination (Martinez, Taddei et al. 1999; Li, Frey et al. 2000; Potter, Klashinsky et al. 2004). Amino acid homologies for individual members of these two groups of proteins range from 60-85% and studies performed in *Citrobacter rodentium*, which also produces attaching and effacing lesions, have suggested functional similarities between the same proteins from various species (Deng, Li et al. 2001). Thus, we expected that there would be significant immunological cross reactivity between Tir and EspA from different STEC serotypes. An $EspA_{O157}$-specific monoclonal antibody was able to cross react with protein of all serotypes with the exception of O103:H2 (Figure 4.1a) whereas there was significantly less cross reactivity and hence more divergence in the protein when a Tir_{O157}-specific monoclonal antibody was used. Further evidence of divergence of these proteins among the serogroups was shown as the bovine antisera from the non-O157 serotypes did not react with the O157 Tir and EspA antigens (Figure 4.6). The Tir_{0157}-specific monoclonal antibody did not react with the Tir protein from any heterologous serotype.

Initially it was not well understood if the lack of cross-reactivity with the Tir_{O157}-specific monoclonal antibody was due to antigenic variation or a lack of production of the Tir protein by non-O157 serotypes. The latter explanation for the lack of cross-reactivity was disproven, since the use of qRT-PCR showed that all serotypes tested produced equivalent levels of the Tir transcripts (Figure 4.3). This was further confirmed as the Tir polyclonal antibody produced using O157 T3SPs was capable of reacting with the Tir protein from all non-O157 serotypes tested (Figure 4.2). Therefore, these results suggested that there is some degree of antigenic variation and not a lack of production of the Tir protein. However, the epitope recognized by Tir_{O157}-specific monoclonal antibody is not known.

A number of Tir deletions were constructed and expressed to identify the location of the epitope recognized by the Tir monoclonal antibody. Using these deletions we were able to pinpoint the possible location of the epitope on all serotypes tested (Figure 4.4, 4.5, and 4.6). Further investigation of the sequence of the epitope shown on Figure 4.7, demonstrates that the differences in antibody reactivity appear to reflect the divergence of the epitopes over time. An obvious variability is observed over the entire amino acid region of the epitope (Figure 4.7B). A single amino acid difference could be enough to prevent the Tir monoclonal antibody from recognizing the epitope in the other Tir proteins from the non-O157 STEC serotypes. A previous study involving evaluation of subunit vaccine components from *Streptococcus uberis* and *Streptococcus dysgalactiae* demonstrated that proteins with 92% identity did not confer cross-protection following immunization due to the presence of non-conserved regions containing the protective epitopes (Fontaine, Perez-Casal et al. 2002). Therefore, it is perhaps not surprising that cross-reactivity did not occur due to the significant variability between the T3SPs, specifically EspA and Tir, of the four serotypes.

In order to quantify the serological relatedness between the T3SPs produced by these four serotypes, antisera were raised in cattle and the degree of cross-serotype reactivity determined using ELISA analysis. There was a remarkable lack of cross serotype reactivity when sera from immunized groups were tested against secreted proteins from the heterologous serotypes (Figure 4.5), suggesting considerable divergence of the epitopes of the secreted proteins presented to the immune system. The sera from the group which received the O26:H11 vaccine did not cross-react significantly with proteins of any other serotype, suggesting it produced proteins which were most divergent. In contrast antisera from O111:NM T3SPs showed significant cross-reaction with all other groups.

The adherence inhibition assay provided a functional method to test the adherence inhibition properties of antiserum raised against a specific antigen (Darfeuille-Michaud, Forestier et al. 1986; Darfeuille-Michaud, Aubel et al. 1990). In addition, the inability to inhibit adherence may be reflected in the ability to inhibit colonization. In this assay the use of a bovine intestinal epithelial cell line would have been preferable, but only primary cell lines which are uncharacterized were available. Thus, HEp-2 cells were used which

are of human origin, but are well characterized and have been used extensively in bacterial adherence assays. A lack of cross-serotype reactivity was observed as the heterologous sera was unable to inhibit adhesion to the same extent as homologous sera, thus confirming the ELISA results. However, the sera against the O111:NM proteins showed the weakest response, while the anti-O157:H7 sera showed the most significant cross-inhibition between all groups. This was surprising, since cattle immunized with the O111:NM antigens showed the greatest cross-serotype reactivity when measured by ELISA, suggesting that the response was due to epitopes not involved in attachment. Another possibility for these conflicting results with STEC O111:NM could be that in an ELISA the antigens tested are significantly more denatured. Unlike the previous experiment, O26:H11 was capable of demonstrating some level of cross inhibition. Overall cross-reactivity using heterologous sera was limited between groups, where inhibition was only clearly evident when homologous serum was tested. However, there are several instances where the levels of serological cross-reactivity with heterologous T3SPs by ELISA or adherence inhibition assay, although less than that seen with sera against the homologous T3SPs, were significantly greater than the corresponding preimmune sera (Figures 4.5 and 4.7). This was also reflected by the lack of cross-reactivity against Tir_{O157} and $EspA_{O157}$ which suggests that other T3SPs were the cross-reactive components. Based on the adherence inhibition assays, the use of serotype O157:H7 antigens as a platform for measuring cross-reactive response appears to be appropriate.

In order to ensure that the adherence inhibition was due to antibody raised against T3SPs and not other components of the bacteria such as cellular debris, LPS and surface proteins, that could have contaminated the supernatant, we absorbed the serum raised against STEC O157 T3SPs with acetone powder made from whole cells from STEC O157. This treatment enabled any antibody present in the sera against contaminating factors to attach to the acetone powder. This powder was then centrifuged and removed from the serum leaving behind antibody raised against other factors present in the supernatant such as T3SPs. The removal of antibody against contaminants was confirmed (Figure 4.9) where a clear reduction was seen with the reactivity with whole cell extract.

The absorbed sera still functioned like the unabsorbed sera in the inhibition assay confirming that the adherence inhibition was a result of antibody against secreted T3SPs.

A number of potential vaccines have been tested for their ability to reduce colonization of cattle by *E. coli* O157:H7. These include formulations containing LPS (Milon, Esslinger et al. 1992; Conlan, Cox et al. 1999; Conlan, KuoLee et al. 2000) as well as intimin (Dean-Nystrom, Gansheroff et al. 2002). While both have met with some success, the serotype-specificity of these molecules suggests that they may have limited utility in preventing colonization by non-O157 strains. Taken together, the data described above suggests that this will also be the case for Type III-secreted proteins produced by STEC serotypes due to the lack of cross-reactivity obtained, suggesting that new approaches to vaccine development will be required if the goal of cross-protection against colonization of cattle by multiple STEC serotypes is to be achieved.

5.0 VACCINATION OF MICE WITH A RECOMBINANT CHIMERIC TIR PROTEIN CONTAINING EPITOPES FROM NON-O157 SEROTYPES

5.1 Introduction

Shiga toxin-producing *Escherichia coli* O157:H7 is a zoonotic pathogen responsible for numerous outbreaks worldwide. Shiga toxin-producing *E. coli* O157:H7 infection can lead to hemorrhagic colitis, resulting in serious complications such as HUS and TTP (Karmali, Petric et al. 1983; Morrison, Tyrrell et al. 1986; Karmali, Petric et al. 2004). Hemolytic-uremic syndrome is a consequence of Shiga toxins produced by STEC, which were previously identified in *Shigella dysenteriae* (Paton and Paton 1998; Kulkarni, Goldwater et al. 2002). Although STEC O157:H7 is the most common serotype in North America, non-O157 serotypes are responsible for frequent global outbreaks (Gonzalez Garcia 2002; Bielaszewska, Zhang et al. 2007). Improved detection and isolation of non-O157 serotypes have identified many non-O157 serotypes as culprits in recent outbreaks, which could have been previously misdiagnosed.

To cause infection, STEC uses a T3SS which allows intimate interaction between an epithelial cell and the bacterium. This interaction results in the formation of attaching and effacing (A/E) lesions, which are the hallmark of STEC infection (Jarvis and Kaper 1996; Gyles 2007). The genes responsible for the secretion apparatus are located on a pathogenicity island called LEE (Elliott, Sperandio et al. 2000). Several effectors on the LEE Island, as well as numerous non-LEE effectors are secreted through the T3SS (Tobe, Beatson et al. 2006). One of the most important and best studied LEE effectors is Tir. The study of Tir began with the identification of the STEC outer membrane adhesion protein called intimin (Jerse, Yu et al. 1990). Initially, it was assumed that intimin bound to a 90 kDa host protein called Hp90. However, in 1997 Kenny and colleagues discovered that the Hp90 was actually a LEE encoded T3SS effector protein, eventually re-named Tir (Kenny, DeVinney et al. 1997). The surprising ability of a bacterial pathogen to carry its own receptor was a trait only observed in A/E pathogens.

Once Tir is translocated through the T3SS, it spontaneously localizes to the plasma membrane of the eukaryotic cell (Kenny, DeVinney et al. 1997; Deibel, Kramer et al. 1998). This protein contains two trans-membrane domains, which form a surface-

exposed extracellular loop responsible for binding the intimin protein, while the N and C termini are implanted within the eukaryotic cell (de Grado, Abe et al. 1999; Hartland, Batchelor et al. 1999; Kenny 1999). The extracellular loop is 104 amino acids long and is critical for intimin binding and pedestal formation (de Grado, Abe et al. 1999). Both Tir and intimin proteins are dimers, allowing each intimin monomer to bind to a monomer of the Tir protein, resulting in an intimate interaction between the bacterium and the host cell (Luo, Frey et al. 2000). The intracellular N and C termini of the Tir protein can recruit and interact with a number of intracellular host and bacterial proteins. These interactions result in actin polymerization and pedestal formation as observed with typical STEC infection.

Ruminants are a natural reservoir of STEC, where they shed the organism asymptomatically year round. Cattle are the most studied animal reservoir due to their close contact with humans and their ability to indirectly infect people through the contamination of water and food products (Caprioli, Morabito et al. 2005). Several vaccines aimed at reducing shedding of STEC, based on LEE proteins, have been tested. The vaccination of pregnant dams using the LEE protein intimin showed promise as it protected against challenge with STEC O157:H7 (Dean-Nystrom, Gansheroff et al. 2002). However, with over 17 distinct variants of STEC identified, this potential vaccine appeared to be serotype specific (Garrido, Blanco et al. 2006). In 2004, Potter and colleagues demonstrated that vaccination of cattle with secreted proteins containing LEE and non-LEE effectors from STEC O157:H7, was able to significantly reduce the number of bacteria shed, as well as the number of shedding animals (Potter, Klashinsky et al. 2004). However, this vaccination using T3SPs also appears to be serotype specific (Asper, Sekirov et al. 2007). Interestingly, vaccination with T3SPs from a Δ*tir* STEC O157 strain did not protect against challenge as effectively as the wildtype strain. This demonstrates that Tir likely plays an important role in protection against STEC O157. The Tir protein has also been shown to be highly immunogenic, and commonly reacts with sera from HUS patients shortly after onset of STEC infection (Li, Frey et al. 2000; Asper, Sekirov et al. 2007).

In this study we constructed, a chimeric STEC Tir protein, containing the STEC O157:H7 Tir protein and several non-O157 immunogenic peptides in order to test its

cross-serotype protective properties. These results could produce a potential candidate for a recombinant cross-protective STEC vaccine.

Materials and Methods

5.2.1 Bacterial strains and growth conditions

Bacterial strains used in this study included STEC EDL933 (O157:H7) (Tarr, Neill et al. 1989), CL101 (O111:NM), CL9 (O26:H11), and N01-2454 (O103:H2) (Karmali, Mascarenhas et al. 2003). Strains were stored at -70^{o}C in 30% glycerol and were grown in LB broth and on LB agar at 37^{o}C. All non-O157 STEC strains were kindly provided by Dr. M. Karmali (Laboratory for Foodborne Zoonoses, Guelph, Ontario).

5.2.2 Production of Type III–secreted proteins and rabbit anti-T3SP polyclonal antibodies

Type III-secreted proteins from all STEC serotypes were prepared as described (Li, Frey et al. 2000). Briefly, overnight cultures of STEC serotypes were diluted 100-fold in M9 minimal media with the addition of 0.4% glucose, 0.1% Casamino acids, 44 mM $NaHCO_2$, 8 mM $MgSO_4$ and 45 mM $KHCO_3$. Cultures were incubated without shaking at 37ºC in a 5% CO_2 environment to an OD_{600} of 0.6-0.8. The bacteria were pelleted by centrifugation and the secreted proteins present in the supernatant were concentrated by precipitation with 10% trichloroacetic acid. One millilitre quantities containing 50 μg of STEC T3SPs, and 30% of Emulsigen® - D (MVP laboratories) were used to subcutaneously immunize female New Zealand white rabbits. The animals received two boosts, three weeks apart, before being euthanized.

5.2.3 Peptide synthesis

Based on the predicted sequence of the Tir protein for STEC serotype O157 (GenBank NC_002655), serotype O103 (GenBank AJ303141), serotype O26 (GenBank AJ277443) and serotype O111 (GenBank AF025311) thirty-mer peptides with five amino acid overlaps were synthesized as described (Fields and Noble 1990) (Table 5.1). All peptides were purified to homogeneity by high-performance liquid chromatography.

Table 5.1. Sequence of STEC O157:H7 Tir and non-O157 Tir peptides used in this study. All peptides are thirty-mers with 5 amino acid overlaps. Underlined peptides in O157 section represent the intimin binding domain. In total seven thirty-mer peptides were constructed for each of the non-O157 STEC serotypes (O26:H11, O103:H2 and O111:NM).

O157	
1-MPIGNLGHNPNVNNSIPPAPPLPSQTDGAG	Tir O157 AA 1-30
2-TDGAGGRGQLINSTGPLGSRALFTPVRNSM	Tir O157 AA 26-55
3-VRNSMADSGDNRASDVPGLPVNPMRLAASE	Tir O157 AA 51-80
4-LAASEITLNDGFEVLHDHGPLDTLNRQIGS	Tir O157 AA 76-105
5-RQIGSSVFRVETQEDGKHIAVGQRNGVETS	Tir O157 AA 101-130
6-GVETSVVLSDQEYARLQSIDPEGKDKFVFT	Tir O157 AA 126-155
7-KFVFTGGRGGAGHAMVTVASDITEARQRIL	Tir O157 AA 151-180
8-RQRILELLEPKGTGESKGAGESKGVGELRE	Tir O157 AA 176-205
9-GELRESNSGAENTTETQTSTSTSSLRSDPK	Tir O157 AA 201-230
10-RSDPKLWLALGTVATGLIGLAATGIVQALA	Tir O157 AA 226-255
11-VQALALTPEPDSPTTTDPDAAASATETATR	Tir O157 AA 251-280
12-ETATRDQLTKEAFQNPDNQKVNIDELGNAI	Tir O157 AA 276-305
13-LGNAIPSGVLKDDVVANIEEQAKAAGEEAK	Tir O157 AA 301-330
14-GEEAKQQAIENNAQAQKKYDEQQAKRQEEL	Tir O157 AA 326-355
15-RQEELKVSSGAGYGLSGALILGGGIGVAVT	Tir O157 AA 351-380
16-GVAVTAALHRKNQPVEQTTTTTTTTTTSA	Tir O157 AA 376-405
17-TTTSARTVENKPANNTPAQGNVDTPGSEDT	Tir O157 AA 401-430
18-GSEDTMESRRSSMASTSSTFFDTSSIGTVQ	Tir O157 AA 426-455
19-IGTVQNPYADVKTSLHDSQVPTSNSNTSVQ	Tir O157 AA 451-480
20-NTSVQNMGNTDSVVYSTIQHPPRDTTDNGA	Tir O157 AA 476-505
21-TDNGARLLGNPSAGIQSTYARLALSGGLRH	Tir O157 AA 501-530
22-GLRHDMGGLTGGSNSAVNTSNNPPAPGSHRFV	Tir O157 AA 526-558
O26	
1-RADPKLWLSLGTIAAGLIGMAATGIAQAVA	Tir O26 AA 218-247
2-AQAVALTPEPDDPITTDPDAAANTAEAAAK	Tir O26 AA 243-272

3-EAAAKDQLTKEAFQNPDNQKVNIDENGNAI	Tir O26 AA 268-297
4-NGNAIPSGELKDDVVAQIAEQAKAAGEQAR	Tir O26 AA 293-322
5-GEQARQEAIESNSQAQQKYDEQHAKREQEM	Tir O26 AA 318-347
6-REQEMSLSSGVGYGISGALILGGGIGAGVT	Tir O26 AA 343-372
7-GAGVTAALHRKNQPAEQTITTRTVVDNQPT	Tir O26 AA 368-397
O103	
1-RADPKLWLSLGTIAAGLIGMAATGIAQAVA	Tir O103 AA 218-247
2-AQAVALTPEPDDPTTTDPDTAASTAEAATK	Tir O103 AA 243-272
3-EAATKDRLTQEAFQDPDKQKVNIDENGNAI	Tir O103 AA 268-297
4-NGNAIPSGELIDDVVAQIAEQAKAAGEQAR	Tir O103 AA 293-322
5-GEQARQEAIESNSQAQKKYDEQHAKREQEM	Tir O103 AA 318-347
6-GEQARQEAIESNSQAQKKYDEQHAKREQEM	Tir O103 AA 343-372
7-GAGVTAALHRKNQPAEQTITTRTVVDNQPT	Tir O103 AA 368-397
O111	
1-RSDPKFWVSIGAIAAGLAGLAATGITQALA	Tir O111 AA 229-258
2-TQALALTPEPDDPTTTDPEQAASAAESATR	Tir O111 AA 254-283
3-ESATRDQLTQEAFKNPENQKVSIDEIGNSI	Tir O111 AA 279-308
4-IGNSIPSGELKDDVVAKIEEQAKEAGEAAR	Tir O111 AA 304-333
5-GEAARQQAVESNAQAQQRYDTQYARRQEEL	Tir O111 AA 304-333
6-RQEELELSSGIGYSLSSALIVGGGIGAGVT	Tir O111 AA 354-383
7-GAGVTTALHRRNQPAEQTTTTTTHTVVQQQ	Tir O111 AA 379-408

5.2.4 The construction of chimeric Tir proteins

Strains EDL933 (O157:H7) (Tarr, Neill et al. 1989), CL101 (O111:NM), CL9 (O26:H11), and N01-2454 (O103:H2) were used as the source of DNA (Karmali, Mascarenhas et al. 2003). The desired DNA was amplified by PCR with unique restriction sites and cloned into the pQE-30 plasmid (Qiagen) for the chimeric 6x HIS-tagged protein (Qiagen) and into the pAA352-Tir plasmid (already has Tir from EDL933 cloned into the vector) for the Leukotoxin::chimeric Tir fusion (LKt::TirO157$_{non\text{-}O157pep}$) protein. Ligations were completed using the Rapid DNA Ligation Kit (Fermentas), and plasmids were chemically transformed into *E. coli* JM105 cells. Primers and restriction sites for DNA fragments cloned can be found in Table 5.2. Glycine and serine amino acid nucleotide sequences were included in the primers to introduce or increase flexibility of the translated protein.

5.2.5 Expression and purification of HIS-tagged chimeric Tir protein.

An overnight LB culture was inoculated at 1:100 into fresh LB + 100 μg/mL ampicillin. Culture was grown at 37ºC with shaking to an OD_{600} of 0.6 and induced for 3 hours by the addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacteria were pelleted and HIS-tagged proteins were purified with Ni-NTA Agarose (Qiagen) under denaturing conditions using the protocol from QIAexpressionist (Qiagen). The purity of proteins was assessed by SDS-PAGE followed by Coomassie Brilliant Blue staining (Laemmli 1970).

5.2.6 Expression and purification of Leukotoxin::chimeric Tir protein fusion

The expression of the LKt::TirO157$_{non\text{-}O157pep}$ *tir* gene was induced by the addition of 5 M IPTG and the protein was purified as insoluble aggregates as described (Gerlach, Anderson et al. 1992). Briefly, after induction bacterial culture was centrifuged and supernatant was discarded. The pellet was re-suspended in a 25% sucrose/50 mM Tris solution and placed at -70ºC for 30 minutes. The cells were then thawed and a lysozyme solution (10 mg lysozyme per mL of 0.25 M Tris pH 8.0) was added to the cells and incubated on ice for 15 minutes. A total of 30 mL of freshly prepared RIPA/TET was added and incubated for 5 minutes. The sample was then sonicated (3 x 30 seconds with

Table 5.2. Oligonucleotide primers used for the amplification of STEC Tir and non-O157 Tir peptides. Nucleotide sequence is from 5' to 3'. AA-cloned amino acids. All four fragments are separated by glycines and serines. F= forward primer; R= reverse primer. Primers were used for PCR for the construction of fragments. Fragments were digested with the appropriate restriction enzymes and cloned into the pQE-30 plasmid (Qiagen) for the chimeric 6x HIS-tagged protein (Qiagen) and into the pAA352-Tir plasmid (already has Tir from EDL933 cloned into the vector) for the LKt::TirO157$_{non-O157pep}$ protein, followed by ligation. Ligations were completed using the Rapid DNA Ligation Kit (Fermentas), and plasmids were chemically transformed into *E. coli* JM105 cells.

Primers	Restriction sites & cloned AA
TirO157-F	KpnI
CGGGGTACCCCTATTGGTAATCTTGGTCATAATCCCAATGTGAATAATTC	
TirO157-R	GSGS-AgeI-PstI
AAAACTGCAGACCGGTGGAGCCAGAACCGACGAAACGATGGGATCCCG	
TirO111-F	AgeI
GGCTACCGGTGAAAGTGCGACAAGAGATCAGTTAACGCAAGAAGCATTCAAG	
TirO111-R	SGSG-SpeI-GS-HindIII
CCCAAGCTTAGAACCACTAGTCCCCGATCCTGATAATTCCTCCTGACGTCTGGCATAC	
TirO26-F	SpeI
GGACTAGTGCACAGGCTGTTGCGTTGACTCCAGAGCCGGATG	
TirO26-R	SSGG-NsiI
CCAATGCATTCCGCCGGATGAAATTGCATTTCCGTTCTCATCG	
TirO103-F	NsiI
CCAATGCATGGGGAACAGGCCAGACAGGAAG	
TirO103-R	HindIII
CCCAAGCTTCATTTCCTGTTCGCGTTTAGC	

1 second pulse, 12 mm probe with max power, Vibra-Cell, Sonics & Materials Inc, Dansbury, USA). The solution was then centrifuged for 20 minutes at 12,000 rpm and the supernatant discarded. The pellet containing the aggregated proteins was solubilized in 4 M guanidine hydrochloride and the concentration of the LKt::TirO157$_{non\text{-}O157pep}$ protein was estimated following SDS-PAGE and Coomassie Brilliant Blue staining using bovine serum albumin standards (BioRad Laboratories) (Laemmli 1970).

5.2.7 SDS-PAGE and Western blot analysis

Proteins were separated by SDS-PAGE and visualized following Coomassie Brilliant Blue staining (Laemmli 1970). Proteins were transferred to a nitrocellulose membrane by electroblotting as described by the manufacturer (BioRad Laboratories). Western blot analysis was carried out as described in Section 3.5. After blocking, the primary STEC Tir O157:H7 monoclonal antibody (Bioniche Life Sciences) was added at a dilution of 1/5000 and incubated for an hour. After washes, the secondary antibody consisting of phosphatase labelled goat-anti-mouse IgG (Kirkegaard & Perry Laboratories) was added at a concentration of 1/2000 and also incubated for an hour at room temperature. After the secondary antibody, the membrane is washed and developed using NBT salt and BCIP (Sigma) (De Jong, Van Kessel-van Vark et al. 1985).

5.2.8 Production of rabbit anti-chimeric Tir protein polyclonal antibodies

One milliliter volumes containing 50 μg of STEC HIS-tagged chimeric Tir protein or LKt::TirO157$_{non\text{-}O157pep}$ fusion protein with the addition of 30% of Emulsigen®-D (MVP laboratories) were used to subcutaneously immunize female New Zealand white rabbits. Rabbits received two boosts, three weeks apart, before being euthanized. Rabbits were bled according to the guidelines provided by the University Council on Animal Care (UCAC), under protocol number 1994-213. The collected blood was centrifuged and the serum was stored at -20ºC until further use.

5.2.9 Enzyme-Linked ImmunoSorbent Assay

Enzyme-Linked ImmunoSorbent Assays using rabbit or mouse polyclonal antibodies against HIS-tagged chimeric Tir protein or LKt::TirO157$_{non\text{-}O157pep}$ protein were completed as described (Li, Frey et al. 2000). Briefly, plates (Immulon 2HB, Thermo) were coated with antigen overnight at 4ºC. Plates were then blocked for 1 hour with nonfat dried milk (CO-OP, Canada) in Tris-Buffered Saline pH 7.6 with the addition of 0.05% Tween-20 (Sigma) (TBST). Serial 4-fold dilutions starting at 1/40 were completed for primary antibody and incubated for 2 hours at room temperature. After 6x H_2O washes, phosphatase-labelled goat-anti-rabbit IgG (Kirkegaard & Perry Laboratories) or phosphatise-labelled goat-anti-mouse IgG (Kirkegaard & Perry Laboratories) at 1/2000 was added and incubated for 1 hour at room temperature, and the alkaline phosphate activity was detected with *p*-nitrophenyl phosphate (PNPP) (Sigma). The absorbance was measured at 405 nm using a BioRad model 1350 microplate reader.

5.2.10 Challenge of mice with STEC serotypes following vaccination with a HIS-tagged chimeric Tir protein

A total of 4 groups of 10 Balb/c mice were used in the vaccination trial. Group 1 and group 3 were vaccinated with 0.1 M PBS and used as placebo groups, while group 2 and group 4 were vaccinated with 0.5 μg per mouse of the HIS-tagged chimeric Tir protein + 30% Emulsigen®- D. All groups received two vaccinations 21 days apart. Fourteen days following the second vaccination, groups 1 and 2 were challenged orally with 10^9 CFU per mL of nal^r *E. coli* O157 EDL933 strain while groups 3 and 4 were challenged with 10^{10} CFU per mL of Amp^r *E. coli* O111:NM strain. Both challenges were delivered in 0.1 M PBS with 20% sucrose. Two days prior to challenge, water was treated with 5 g/L of streptomycin to remove intestinal flora. Mice were also deprived of food and water 18 hours prior to challenge. Fecal samples were collected every 2 days for 2 weeks after challenge to measure STEC shedding.

On average, 0.1 gram of fecal matter was collected from each mouse (3-4 pellets) and placed in an Eppendorf tube. One milliliter of LB broth was added to the tube and incubated at room temperature for 3 hours to allow pellet to soften. The tubes were then vortexed to allow the dispersal of pellets. For group 1 and 2, samples were diluted in PBS

to 10^{-6} and plated onto CT-SMAC agar plates (Mackonkey agar + cefiximine 0.05 mg/L + tellurite 2.5 mg/L + nalidixic acid 15 mg/L). For group 3 and 4, samples were diluted in PBS to 10^{-8} and plated onto LB-Amp agar plates (ampicillin 10 mg/L). All plates were incubated at 37ºC for 18 hours.

5.3 Results

5.3.1 Identification of immunogenic STEC O157 and non-O157 Tir epitopes

A total of twenty two thirty-mer peptides were constructed for the STEC O157 Tir protein. These peptides were tested with sera raised against T3SPs from STEC serotypes (O157:H7, O111:NM, O26:H11 and O103:H2) to measure their immunogenicity. Shiga toxin-producing *Escherichia coli* O157-specific sera showed the highest level of reactivity and reacted with the most peptides (Figure 5.1). Shiga toxin-producing *Escherichia coli* O103-specific sera also reacted with a large number of peptides while O26- and O111-specific sera showed limited reactivity with the STEC O157 Tir peptides. Overall the homologous sera showed the greatest reactivity.

For each of the non-O157 STEC Tir proteins seven, thirty-mer peptides for the intimin binding region were designed and constructed. All peptides were tested against O26-, O103-, O111- and O157-specific sera. With all peptides (excluding O26) the homologous sera demonstrated the highest levels of reactivity and was capable of reacting with most peptides (Figure 5.2). For the peptides from O26 and O103 serotypes, peptide number three was recognized by all sera tested, while O111 and O157 peptides demonstrated limited reactivity from heterologous sera. In contrast, the STEC O26 peptides showed limited reactivity with the homologous sera; where reactivity with peptide 3 was lower for O26-specific sera than for the O103-, O111- and O157-specific sera tested. The sera tested with peptides from STEC O103, O111 and O157 were able to identify individual peptides, which are only recognized by homologous sera. These include peptide 5 for O103, peptides 3, 4 and 5 for O111 and peptide 3 for O157. The limited reactivity with the O26 peptides could be a result of the titer instead of the reactivity against the peptide and could only be properly confirmed by the measure of total IgG.

Interestingly, the pattern of recognized peptides for serotype O26 and O103 was nearly identical with the exception of peptide 5 for O103 with all sera tested. The selected non-O157 immunogenic peptides for the construction of the chimeric protein are listed in Table 5.2. These consist of peptide 5 from O103, peptides 2 and 3 from O26 and peptides 3, 4 and 5 from O111. This is shown in Figure 5.3.

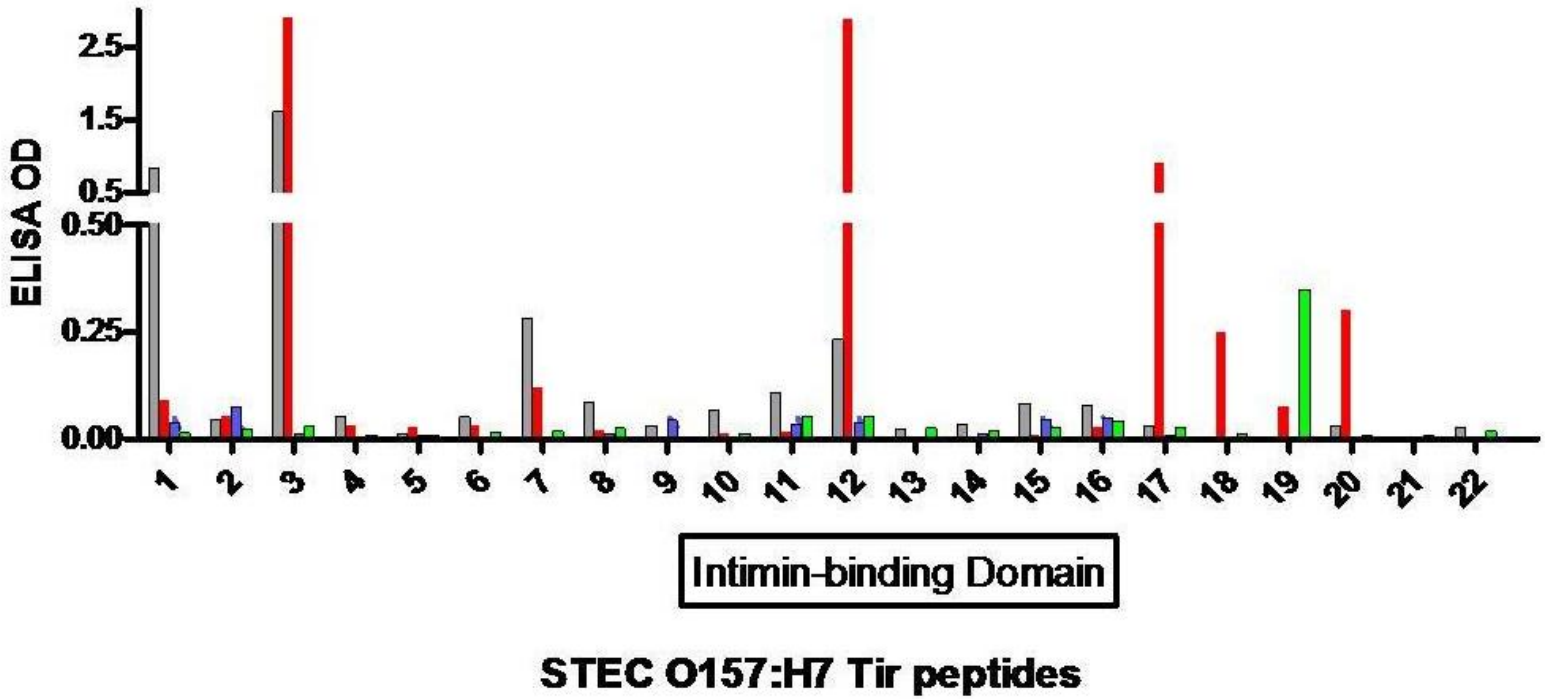

ELISA OD
2.5
1.5
0.5
0.50
0.25
0.00
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Intimin-binding Domain
STEC O157:H7 Tir peptides

Figure 5.1. STEC O157:H7 Tir epitope mapping. Twenty two thirty-mer peptides with 5 amino acid overlaps were constructed for the STEC O157:H7 Tir protein. Rabbit anti-non O157 STEC T3SPs was tested for reactivity against STEC O157:H7 Tir protein thirty-mer peptides.  Anti-O157 T3SPs rabbit sera;  Anti-O26 T3SPs rabbit sera;  Anti-O103 T3SPs rabbit sera;  Anti-O111T3SPs rabbit sera.

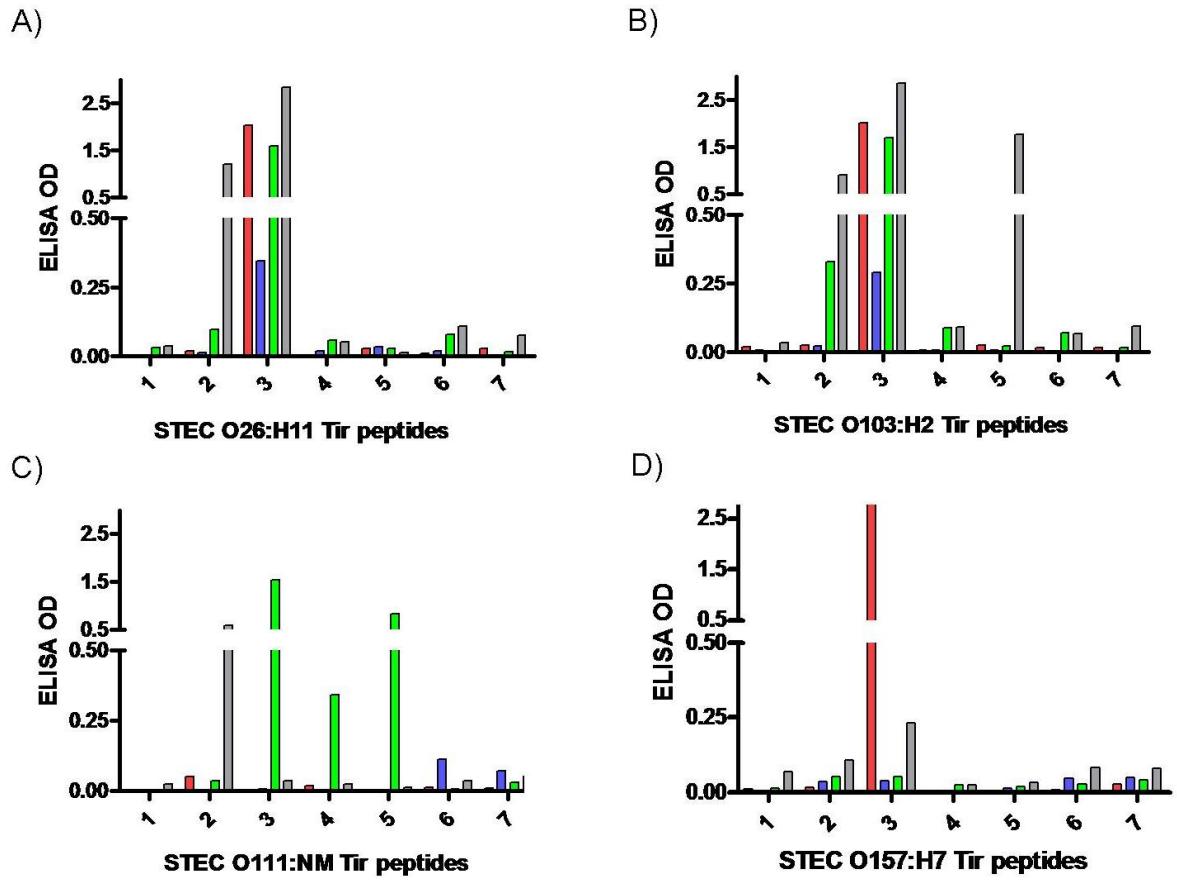


Figure 5.2. Cross-reactivity of STEC polyclonal antibodies against STEC non-O157 peptides. Seven thirty-mer peptides were constructed for each of the non-O157 STEC serotypes (O26:H11, O103:H2 and O111:NM). A) STEC O26:H11 peptides. B) STEC O103:H2 peptides. C) STEC O111:NM peptides. D) STEC O157:H7 peptides Anti-O157 T3SPs rabbit sera; Anti-O26 T3SPs rabbit sera; Anti-O103 T3SPs rabbit sera; Anti-O111T3SPs rabbit sera.

Table 5.3. Designated peptides to be fused to with STEC O157:H7 Tir protein. A) Selected peptides**.** Chosen peptides are noted with an X and are as follows: peptide 5 for STEC O103; peptide 2 and 3 for STEC O26; peptide 3 to 5 for STEC O111. B) Sequence of selected peptides.

A)

	***STEC* non-O157 peptide targets**		
	Serotypes		
Peptides	**O103:H2**	**O26:H11**	**O111:NM**
1			
2		X	
3		X	X
4			X
5	X		X
6			
7			

X= Selected peptides to be used in the chimeric Tir protein

B)

Selected Peptides	**Sequence (Amino Acid)**
Peptide 5 (O103)	GEQARQEAIESNSQAQKKYDEQHAKREQEM
Peptide 2 (O26)	AQAVALTPEPDDPITTDPDAAANTAEAAAK
Peptide 3 (O26)	EAAAKDQLTKEAFQNPDNQKVNIDENGNAI
Peptide 3 (O111)	ESATRDQLTQEAFKNPENQKVSIDEIGNSI
Peptide 4 (O111)	IGNSIPSGELKDDVVAKIEEQAKEAGEAAR
Peptide 5 (O111)	GEAARQQAVESNAQAQQRYDTQYARRQEEL

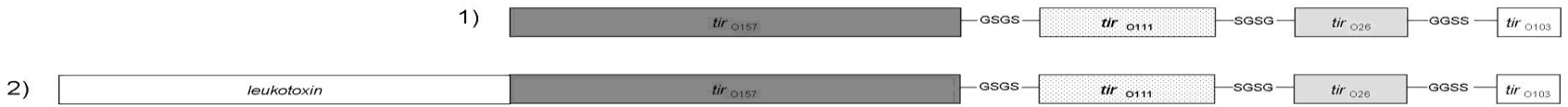

1)
tir O157
GSGS
tir O111
SGSG
tir O26
GGSS
tir O103
2)
leukotoxin
tir O157
GSGS
tir O111
SGSG
tir O26
GGSS
tir O103

Figure 5.3. Construction of Tir chimeric protein. Diagram of fused chimeric Tir proteins**.** 1) Fragments fused with STEC O157:H7 Tir protein. 2) Fragments fused with STEC O157:H7 Tir protein and Leukotoxin. Individual fragments cloned and location of glycines (G) and serines (S). tir_{O157}- The entire tir gene from O157:H7. tir_{O111}- 240 bases from the STEC O111 *tir* gene which transcribes peptide 3 to 5 from Table 5.2. tir_{O26}- 165 bases from the STEC O26 *tir* gene which transcribes peptide 2 and 3 from Table 5.2. tir_{O103}- 90 bases from the STEC O103 *tir* gene which transcribes peptide 5 from Table 5.2. Glycine and serine amino acids were cloned in between fragments to allow for proper flexibility of chimeric protein.

5.3.2 STEC O157:H7 Tir monoclonal antibody tested against chimeric Tir proteins

The monoclonal antibody for the STEC O157 Tir protein reacted with all proteins tested, including the STEC O157 Tir protein, the chimeric Tir protein, the LKt::TirO157 fusion protein and the LKt::TirO157$_{\text{non-O157pep}}$ protein (Figure 5.4). A clear size difference was observed between the wildtype Tir protein and the chimeric Tir protein where the chimeric protein had a higher molecular weight in a polyacrylamide gel, due to the inclusion of the non-O157 peptides. A similar profile is observed with the LKt::TirO157$_{\text{non-O157pep}}$ protein (Figure 5.4).

5.3.3 Antibody response of vaccinated rabbits to fused non-O157 Tir peptides

Rabbit polyclonal antibody raised against the constructed recombinant proteins was tested in an ELISA against the chimeric Tir protein and the LKt::TirO157$_{\text{non-O157pep}}$ protein. Both sera responded well to their respective proteins, where the chimeric Tir protein resulted in nearly a four log difference, while the LKt::TirO157$_{\text{non-O157pep}}$ protein showed a two log difference when compared to the preimmune sera (Figure 5.5). Both rabbit sera were also tested against the individual components of the chimeric Tir proteins (Figure 5.6). These components consisted of three STEC O111 peptides (PEP 3-5), two STEC O26 peptides (PEP 2 & 3) and one STEC O103 peptide (PEP 5) (Table 5.2) and the native STEC O157 Tir proteins. Both sera, consisting of antisera specific for the chimeric Tir protein, and antisera specific for the LKt::TirO157$_{\text{non-O157pep}}$ protein, responded well to all peptides tested in comparison to the preimmune sera. A significant response was also seen when the sera were tested against the STEC O157 Tir protein. Overall, the sera raised against the chimeric Tir protein showed greater reactivity to each individual peptide than the sera against the LKt::TirO157$_{\text{non-O157pep}}$ protein. The difference is highlighted with the response observed with peptide 5 from STEC 103 and peptide 5 from STEC O111. The peptide SN11 was used as a negative control where the rabbit titers were the same for all sera tested.

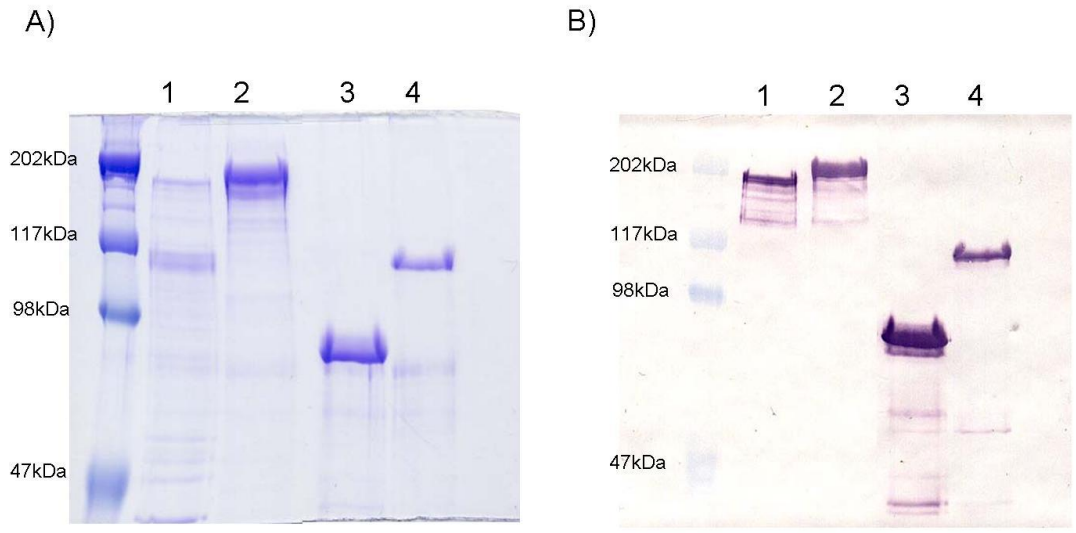


Figure 5.4. Immunological reactivity of monoclonal STEC O157 Tir antibody against purified chimeric Tir protein and LKt::TirO157$_{non\text{-}O157pep}$ protein. All proteins were analysed on a 12% SDS-PAGE gel. A) Coomassie stained gel. B) Western blot was completed using an STEC O157:H7 anti-Tir monoclonal antibody. 1- LKt::TirO157 protein; 2- LKt::TirO157$_{non\text{-}O157pep}$ protein; 3- Tir protein; 4- Tir protein::non-O157 Tir peptides.

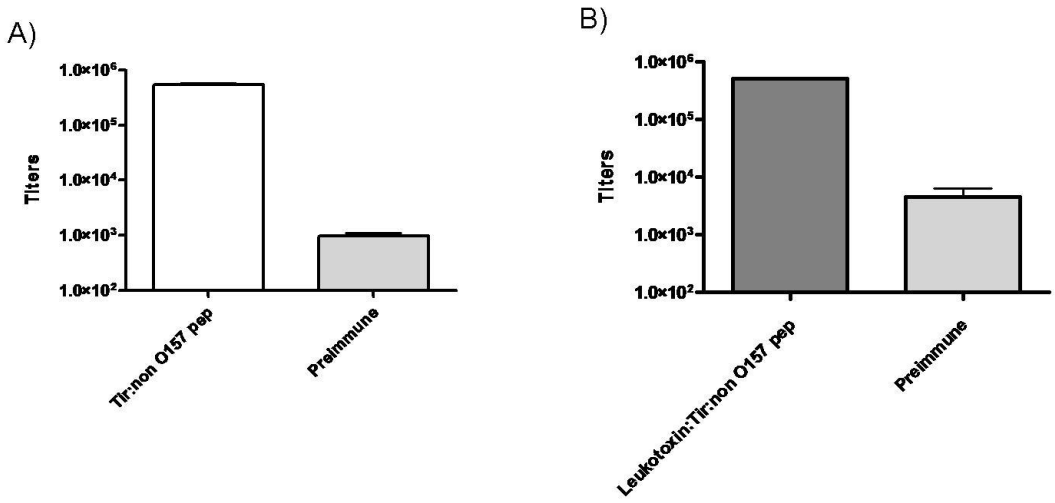


Figure 5.5. Antibody titers of vaccinated rabbits against chimeric Tir protein and Leukotoxin::Chimeric Tir fusion protein. A) Sera tested against chimeric Tir protein containing non-O157 peptides B) Sera tested against Leukotoxin::Chimeric Tir fusion protein containing non-O157 peptides. Rabbits were vaccinated with 50 μg of recombinant chimeric proteins followed by two boosts, three weeks apart before being euthanized.

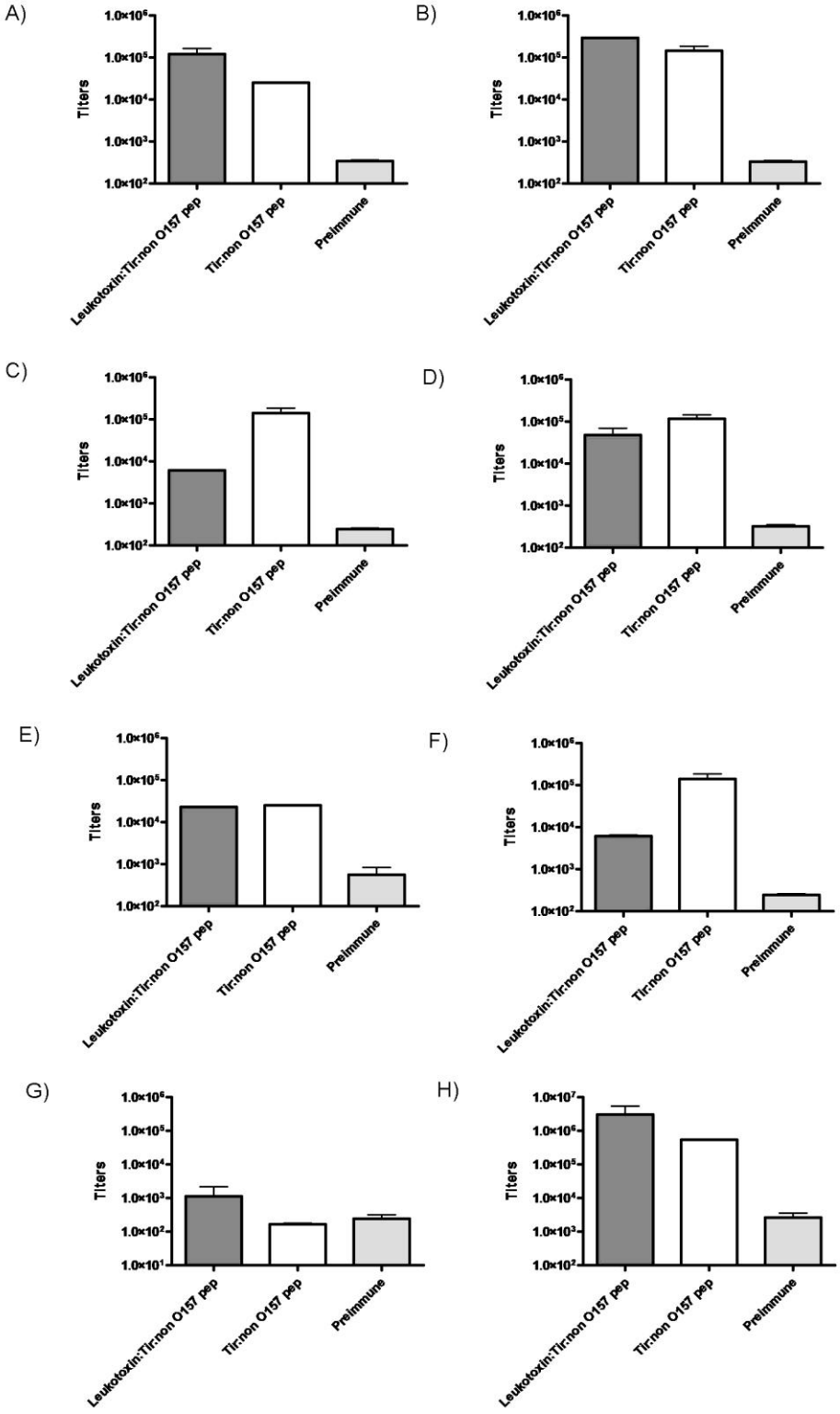

A)
B)
C)
D)
E)
F)
G)
H)
Titers
Leukotoxin:Tir:non O157 pep
Tir:non O157 pep
Preimmune

Figure 5.6. Antibody titers of vaccinated rabbits against individual non-O157 peptides within chimeric Tir protein and Leukotoxin::Chimeric Tir fusion protein. A) Peptide 1 [O26]. B) Peptide 2 [O26]. C) Peptide 5 [O103]. D) Peptide 3 [O111] E) Peptide 4 [O111]. F) Peptide 5 [O111]. G) peptide SN11 H) STEC O157 Tir protein. Rabbits were vaccinated with 50 μg of recombinant chimeric proteins followed by two boosts, three weeks apart before being euthanized.

5.3.4 Fecal shedding of STEC serotypes after vaccination with a HIS-tagged chimeric Tir protein

Mice were vaccinated with a HIS-tagged chimeric Tir protein and challenged with either STEC O157 or STEC O111 to determine if a reduction in shedding could be observed after challenge. Placebo groups were also challenged.

Overall there appeared to be no significant difference between the placebo groups and the vaccinated groups following challenge with either STEC O157 or STEC O111 (Figure 5.7 and Figure 5.8). Throughout the 14 days, both groups vaccinated with the HIS-tagged chimeric Tir protein shed equivalent amounts compared to the placebo groups. However, differences between the groups challenged with STEC O157 and STEC O111 were observed. The level of shedding of STEC O157 decreased significantly throughout the trial from roughly 10^9 to 10^5 (Figure 5.7). In contrast shedding by the mice challenged with STEC O111 appeared to fluctuate within two logs of the initial shedding observed on day 2 (Figure 5.8).

Statistical analysis of mouse fecal shedding was completed by incorporating repeated measures using the ANOVA test where the data were summed over time. The sums which were not normally distributed were log transformed and one-way ANOVA test was used followed by Tukey's comparison of means test. Over the duration of the study there was no significant difference between the groups.

5.3.5 Immunological response of mice to vaccination with HIS-tagged chimeric Tir protein

Sera collected from the mice involved in the shedding trial were used in an ELISA to test if the mice vaccinated with HIS-tagged chimeric Tir protein seroconverted. Overall mice used in both trials in Figure 5.7 and Figure 5.8 were able to successively seroconvert after vaccination (Figure 5.9). The mice in the placebo groups did not produce significant titers since they were not vaccinated with the chimeric Tir protein. The lowest titers were observed on day 0 while the highest on day 48.

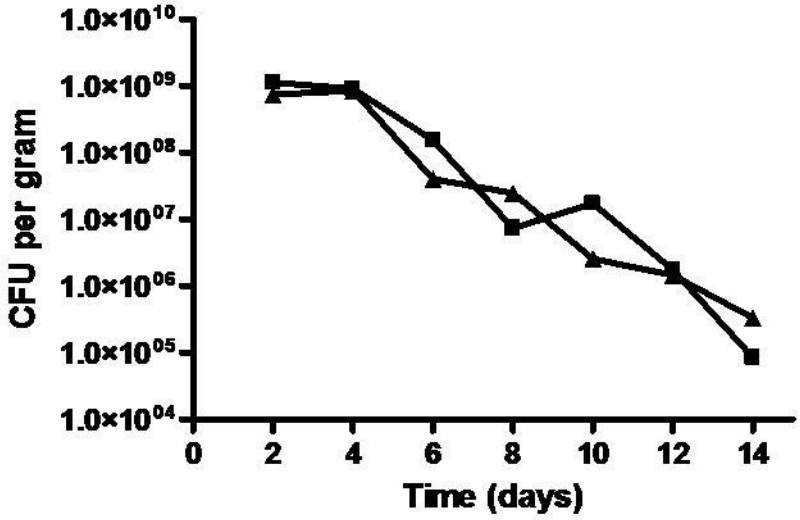


Figure 5.7. Challenge of mice with STEC O157 following immunization with HIS-tagged chimeric Tir protein. Two groups of 10 Balb/c mice were vaccinated with 0.5 µg per mouse. (■) Group 1 received PBS (placebo group); (▲) Group 2 was vaccinated with chimeric Tir protein + 30% Emulsigen®- D. Both groups received two vaccinations 21 days apart. Two weeks following the last vaccination all mice were challenged with 100µL oral dose of 10^9 CFU per mL of Nal^r *E. coli* O157 strain in 20% sucrose. Two days prior to challenge, water was treated with 5 g/L of streptomycin to remove intestinal flora. Mice were also deprived of food and water 18 hours prior to challenge. Fecal samples were collected every 2 days for 2 weeks to investigate STEC shedding following vaccinations.

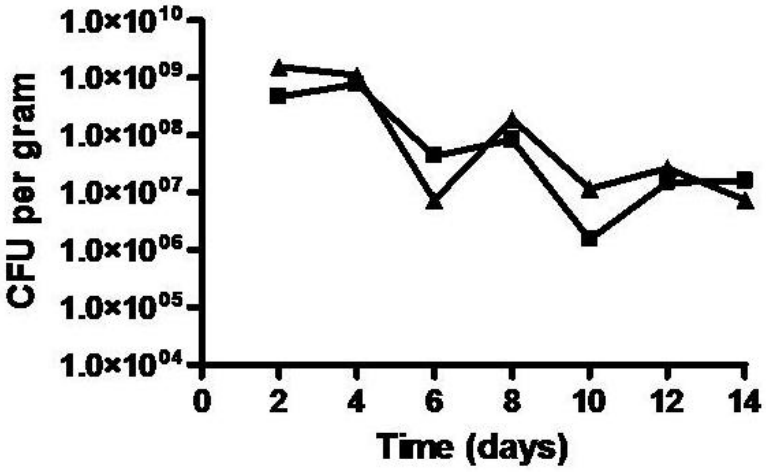
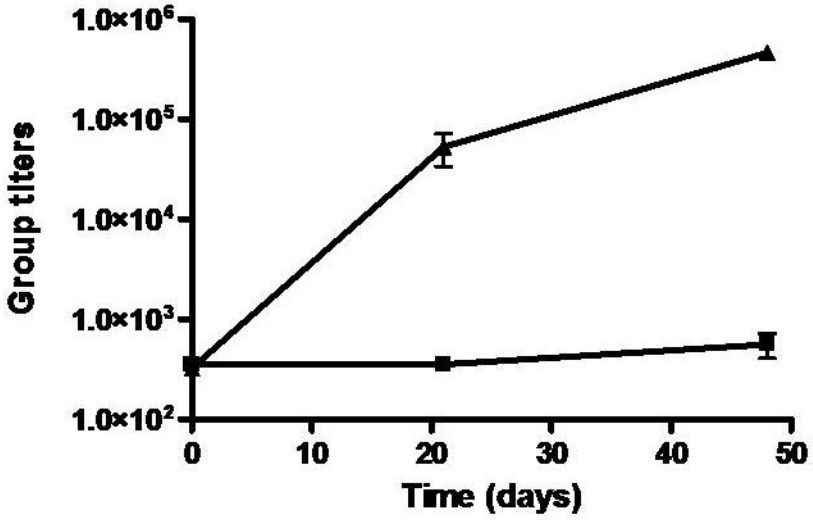


Figure 5.8. Challenge of mice with STEC O111 following immunization with HIS-tagged chimeric Tir protein. Two groups of 10 Balb/c mice were vaccinated with 0.5 µg per mouse. (■) Group 1 received PBS (placebo group); (▲) Group 2 was vaccinated with a chimeric Tir protein + 30% Emulsigen®- D. Both groups received two vaccinations 21 days apart. Two weeks following the last vaccination all mice were challenged with 100µL oral dose of 10^{10} CFU per mL of Amp^r *E. coli* O111 strain in 20% sucrose. Two days prior to challenge, water was treated with 5 g/L of streptomycin to remove intestinal flora. Mice were also deprived of food and water 18 hours prior to challenge. Fecal samples were collected every 2 days for 2 weeks to investigate STEC shedding following vaccinations.

A)



B)

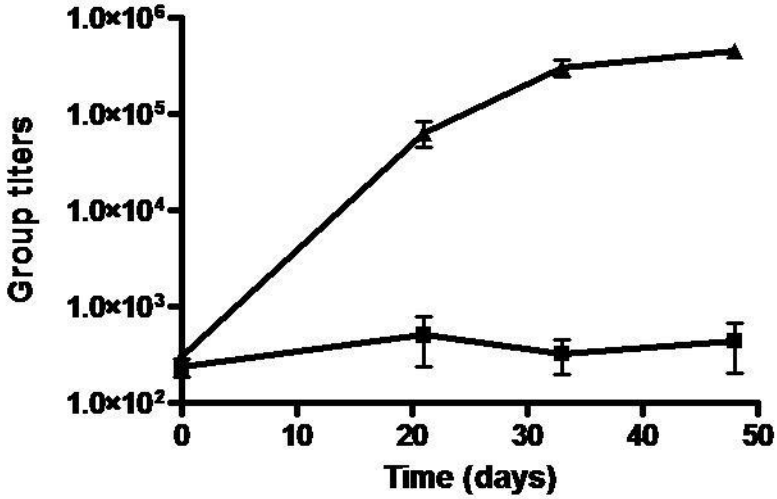


Figure 5.9. Serological responses following vaccination of mice with a HIS-tagged chimeric Tir. The response against the HIS-tagged chimeric Tir was measured by ELISA as in Section 5.2.9. A) Titers from mice challenged with STEC O157; B) Titers from mice challenged with STEC O111 (■) Placebo, (▲) chimeric Tir protein. Vaccinations were given on day 0 and day 21.

5.4 Discussion

Shiga toxin-producing *E. coli* is a zoonotic pathogen responsible for worldwide outbreaks, resulting in complications such as TTP and HUS. At present, HUS is the leading cause of renal failure in children. Shiga toxin-producing *E. coli* utilize a T3SS to attach to and colonize intestinal epithelial cells of infected hosts. The hallmark of an STEC infection is the development of A/E lesions, as result of the intimate interaction between the intimin protein and the secreted effector Tir.

Several studies have reported that Tir is highly immunogenic and capable of producing high antibody titers when used as an antigen for vaccination (Asper, Sekirov et al. 2007). Interestingly, human sera collected from HUS patients, shortly after the onset of an STEC infection, reacted strongly with the Tir protein from STEC O157:H7 (Li, Frey et al. 2000). These results demonstrate that Tir could be a potential candidate for a recombinant STEC vaccine. To further support this hypothesis, Potter and colleagues demonstrated that the vaccination of cattle with T3SPs from a wildtype O157 strain was able to reduce the shedding and protect cattle from challenge with STEC O157:H7 significantly better than T3SPs harvested from a Δ*tir* STEC O157 strain (Potter, Klashinsky et al. 2004).

A number of vaccines have been tested based on proteins translated from the LEE Pathogenicity Island. The STEC receptor, intimin, was shown to protect pregnant dams against challenge with *E. coli* O157:H7 (Dean-Nystrom, Gansheroff et al. 2002). However, due to the many identified variants of this protein, protection would be serotype specific. Several other LEE encoded proteins have also been tested such as EspB (unpublished results) and EspA (Dziva, Vlisidou et al. 2007), which resulted in minimal protection against STEC O157:H7 challenge. At present, the Tir protein is a component of the STEC O157:H7 vaccine called Econiche® (Bioniche Life Sciences) which is based on T3SPs. Although this vaccine works efficiently against STEC O157:H7 challenge, it appears to be serotype specific (Asper, Sekirov et al. 2007). To our knowledge, there is no STEC vaccine or study which reports the ability to cross-protect against a number of STEC serotypes.

In this study we investigated the protective properties of a chimeric Tir protein against STEC serotypes. To construct a cross-protective protein, we initially identified a

number of immunogenic peptides containing epitopes unique to a particular serotype (Figure 5.2 and Table 5.2). These peptides are unique because they are only recognized by the homologous sera. A candidate peptide represents an immunogenic epitope which is unique to a particular serotype and distinguishes it from the other serotypes.

Using thirty-mer peptides constructed to the entire Tir protein from STEC O157:H7, we also identified several peptides which contain immunogenic epitopes throughout the entire O157 Tir protein. Previously it was understood that the Tir protein was immunogenic (Asper, Sekirov et al. 2007). However, the location of the actual immunogenic epitopes had not been identified. Many of these epitopes are also unique since non-O157 antisera against T3SPs (which includes Tir) did not recognize many of the peptides from the O157 Tir protein. In fact, only antisera against T3SPs from STEC O103 demonstrated any considerable reactivity. Another explanation for the difference in specificity could result from the length of the peptides used. All the peptides were thirty-mers with five amino acid overlaps. It is believed that a common epitope ranges from 5 to 15 amino acids (Flower 2005). One must assume that based on sequence lengths involving the addition or deletion of amino acids throughout the Tir sequence of STEC serotypes, some epitopes could have landed in between the 5 amino acid overlaps. This would result in a false negative since the epitope would be split. Overall, these results suggest that there is a considerable level of diversity among the Tir protein specifically the intimin binding domain from STEC serotypes.

All non-O157 peptides constructed for this study were selected from the intimin binding domain. This segment was selected because it is the only region of the Tir protein which is exposed on the outside of the infected intestinal epithelial cell, and is also the intimin binding site. It is possible that by raising an antibody response against this region, it may possibly bind and block the intimate attachment between Tir and intimin, which is critical for the colonization and the development of A/E lesions. After the identification of a number of unique immunological peptides from serotypes other than O157, we constructed a chimeric Tir protein and were able to demonstrate that sera from vaccinated rabbits reacted with each individual peptide, and the entire Tir protein from the STEC O157 serotype (Figure 5.5 and Figure 5.6). This provided evidence that the chimeric

protein had the potential to be a vaccine candidate that would confer protection against a number of serotypes.

Initially two chimeric proteins were constructed. They were identical except that one was fused to leukotoxin. Leukotoxin is the *lktA* gene product from *Mannheimia haemolytica* which has been shown to have adjuvant properties (Hughes, Campos et al. 1992). The fusion of leukotoxin with other proteins has shown to provide a simple antigen-adjuvant formulation that not only acts at the same site as the antigen, but can also target antigen presenting cells (Hughes, Campos et al. 1992; Hughes, Campos et al. 1994). In spite of this both proteins produced significant titers in vaccinated rabbits, the HIS-tagged chimeric Tir protein was selected for the mice vaccination trial because of superior overall titers to the individual non-O157 peptides. Therefore in this case the leukotoxin did not confer immunogenicity

The STEC O157 Tir protein has been shown to be highly immunogenic. This protein is also believed to play a protective role since T3SPs from a STEC O157 Tir mutant did not protect against challenge as well as the T3SP from the wildtype (Potter, Klashinsky et al. 2004; Asper, Sekirov et al. 2007). However, in this study, we have shown that the vaccination of mice with a HIS-tagged chimeric Tir protein which includes the entire STEC O157 Tir protein and a number of non-O157 peptides containing immunogenic epitopes does not protect against STEC O157 and STEC O111 challenge in the streptomycin-treated mice model. The lack of protection was not due to failure to recognize the protein as mice vaccinated with the HIS-tagged chimeric protein seroconverted (Figure 5.9). However, as described, similar results have been observed with other LEE proteins such as EspA, EspB and intimin, as well as STEC colonization factors such as Efa1, where a strong antibody titer was induced, but protection was not observed (Dziva, Vlisidou et al. 2007; van Diemen, Dziva et al. 2007).

Based on the limited protection following vaccination with the HIS-tagged Tir protein, one can speculate that the protection seen with the Econiche® (Bioniche Life Sciences) vaccine could result from the cumulative effect of a number of immunogenic secreted proteins present in the bacterial supernatant used for vaccination. To further test this hypothesis, the identification of immunogenic proteins present in the supernatant need to be identified.

6.0 SEROLOGICAL RESPONSE OF SHIGA-TOXIN PRODUCING ESCHERICHIA COLI TYPE III SECRETED PROTEINS IN VACCINATED RABBITS, NATURALLY INFECTED CATTLE AND HUMAN SERA

6.1 Introduction

Shiga toxin-producing *Escherichia coli* is an important zoonotic pathogen responsible for hemorrhagic colitis, TTP and HUS (Karmali, Petric et al. 1983; Morrison, Tyrrell et al. 1986; Karmali, Petric et al. 2004). Hemolytic-uremic syndrome is attributed to the action of Shiga toxins (Stx1 and Stx2), initially identified in *Shigella dysenteriae*, produced by STEC either alone or in combination, (Paton and Paton 1998; Kulkarni, Goldwater et al. 2002). Hemolytic-uremic syndrome is also the leading cause of acute renal failure in children worldwide.

The most common STEC serotype in North America is O157, where an estimated 73,000 illnesses occur each year in the United States resulting in 2000 hospitalizations and 60 deaths (Mead, Slutsker et al. 1999). The most frequent non-O157 serotypes responsible for disease are O26, O103, O111 and O145 with numerous outbreaks reported worldwide. In Denmark it is estimated that 68% of STEC infections resulted from non-O157 serotypes (Nielsen, Scheutz et al. 2006). It is also estimated that 58% of all cases in Argentina, which has the highest reported frequency of HUS worldwide, result from non-O157 serotypes (Lopez, Diaz et al. 1989; Su and Brandt 1995).

Cattle are the main reservoir for STEC where the organism is asymptomatic, resulting in year round shedding. Colonization of both cattle and human hosts is mediated through the action of effector molecules secreted through a T3SS (Jarvis and Kaper 1996; Gyles 2007). These effectors contribute to the formation of (A/E) lesions, which are the hallmark of STEC infection (Jerse, Gicquelais et al. 1991). The genes which express the structural proteins of the T3SS and many of its effectors are located on a pathogenicity island called LEE (Elliott, Sperandio et al. 2000). Many of these proteins such as Tir , EspA, EspB and EspD are critical for the virulence of STEC (Gyles 2007). The recent discovery of non-LEE effectors such as NleA, TccP and NleB, whose genes are located in small pathogenicity islands and prophages, emphasize the important role effectors play

in the colonization and virulence of STEC (Gruenheid, Sekirov et al. 2004; Kelly, Hart et al. 2006; Tobe, Beatson et al. 2006; Roe, Tysall et al. 2007).

A number of experimental vaccines based on LEE proteins have been tested. The vaccination of pregnant dams using intimin from STEC O157 protected suckling piglets against challenge (Dean-Nystrom, Gansheroff et al. 2002), yet a cross-protective vaccine based on this protein would be challenging, as over 17 serologically distinct variants have been identified (Garrido, Blanco et al. 2006). Potter and colleagues demonstrated that vaccination with secreted proteins from STEC O157:H7 was able to significantly reduce the number of bacteria shed, as well as the number of shedding animals in an experimental setting (Potter, Klashinsky et al. 2004). However, vaccination using T3SPs appears to be serotype specific (Asper, Sekirov et al. 2007). At present, the repertoire of T3SPs in the supernatant which are harvested for vaccination, as well as their immunogenic properties, are unknown. In a natural infection, it is also unclear which T3SPs are secreted by STEC and recognized by cattle and human hosts.

In this study we have cloned and expressed the genes coding for 66 structural and effector proteins which include 37 LEE-encoded proteins and 29 non-LEE effectors to assess their immunological cross-reactivity using sera from vaccinated and naturally infected animals as well as human sera from HUS patients. These studies could identify potential candidates for a recombinant STEC vaccine.

6.2 Materials and Methods

6.2.1 Bacterial strains and growth conditions

Bacterial strains used in this study included *E. coli* EDL933 (O157:H7) (Tarr, Neill et al. 1989), CL101 (O111:NM), CL9 (O26:H11), and N01-2454 (O103:H2) (Karmali, Mascarenhas et al. 2003). Strains were stored at -70^{o}C in 30% glycerol and were grown in LB agar and LB broth at 37^{o}C. All non-O157 STEC strains were kindly provided by Dr. M. Karmali (Laboratory for Foodborne Zoonoses, Guelph, Ontario).

6.2.2 Cloning of LEE and non-LEE genes

The STEC O157:H7 strain EDL933 was used as the source of DNA. The desired region of chromosomal DNA was amplified by polymerase chain reaction (PCR), allowing for the introduction of unique restriction sites cloned into the pQE-30 plasmid (Qiagen) for 6x HIS-tagged proteins (Qiagen) and the pGEX-5X-1 plasmid for Glutathione S-transferase **(**GST)-fused proteins. Ligations were completed using the Rapid DNA Ligation Kit as described by the manufacturer (Fermentas). Plasmids were chemically transformed into *E. coli* JM105 cells (pQE-30) and *E. coli* BL21 cells (pGEX-5X-1). Primers and restriction sites for genes cloned can be found on Table 6.1.

6.2.3 Expression and purification of HIS-tagged LEE and non-LEE proteins

An overnight LB culture was inoculated at 1:100 into fresh LB supplemented with ampicillin (100 μg/mL). The culture was grown at 37ºC with shaking to an OD_{600} of 0.6, and induced for 3 hours with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacteria were pelleted and HIS-tagged proteins were purified with Ni-NTA Agarose under denaturing conditions using the protocol from QIAexpressionist (Qiagen). The purity of proteins was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue staining (Laemmli 1970).

Table 6.1. Oligonucleotide primers used for the amplification of LEE and non-LEE genes. Nucleotide sequence is from 5' to 3'. Restriction sites incorporated into the primers are listed. *=GST fused genes.

Genes		Sequence	Restriction site
LEE genes			
ler	F	CGCGGATCCCGGAGATTATTTATTATGAATATGGAAAATAATTCAC	BamHI
	R	CCCAAGCTTTTAAATATTTTTCAGCGGTATTATTTCTTCTTCAGTGT	HindIII
orf2	F	CGCGGATCCATAACGATAACTGAGCTGGAAGATG	BamHI
	R	CCCAAGCTTCTATTTATTATTAATCCTGATTCGC	HindIII
cesA/B	F	CGCGGATCCAGTATTGTGAGCCAAACAAGAAATAAAG	BamHI
	R	CCCAAGCTTTCATACTATTTTTCTATTATTTCTATTCCG	HindIII
orf4	F	CGCGGATCCACAATTTTTAATAAAATAGAC	BamHI
	R	CCCAAGCTTTCATAAAGTTTCATAAGGC	HindIII
orf5	F	CGCGGATCCCTTACAGAAGATATCATACCAGAGG	BamHI
	R	CCCAAGCTTTCATTCCTGAATAATGCTAAG	HindIII
*escS**	F	CGCGGATCCCC GTTATCGGTATTATTATTAGTCTGG	BamHI
	R	ACGCGTCGACTTAGCCGTTCACCTTCGGAATC	SalI
escT	F	CGCGGATCCAATGAGATAATGACGGTCATAGTATC	BamHI
	R	CCCAAGCTTTCACTCATTAATCATGCTCGGTAAC	HindIII
rorf13	F	CGCGGATCCAAAAAAATAATACTGAGCATCATTCTC	BamHI
	R	CGCGGATCCAAAAAAATAATACTGAGCATCATTCTC	HindIII
grlR	F	CGCGGATCCATTATGAAGGATGGCATCTATAGC	BamHI
	R	CCCAAGCTTTTATTTTAAATAAACTTGTGGCATTCCTGTG	HindIII
grlA	F	CGCGGATCCGAATCTAAAAATAAAAATGGCGAC	BamHI
	R	CGCGGATCCGAATCTAAAAATAAAAATGGCGAC	HindIII
cesD	F	CGCGGATCCAGCAGGAAATTTAGCTCTCTAG	BamHI
	R	CCCAAGCTTTTACTCTGTATTACCTAAC	HindIII
escC	F	CGCGGATCCAAAAAAATAAGTTTTTTTATTTTTACAGCACTATTTTGCTGCAGTGCACAAGCTGCCCC	BamHI

	R	CCCAAGCTTTTATTCGCTAGATGCAGATTTTATCGGGGTTGCTTTAATTAAAAAGAGTCGAACAAC	HindIII
sepD	F	CGCGGATCCAACAATAATAATGGCATAGCAAAGAATG	BamHI
	R	CCCAAGCTTTTACACAATTCGTCCTATATCAGAAAAC	HindIII
escJ	F	CGCGGATCCAAAAAACACATTAAAAACCTTTTTTTATTGGCTGC	BamHI
	R	CCCAAGCTTTTACCCGTCCTGTCCTGAGGATGACTTGATAACAAC	HindIII
orf8	F	CGCGGATCCGATGTATTATGCCCTTGCCTCTTTCATAAAAAG	BamHI
	R	CGCGGATCCGATGTATTATGCCCTTGCCTCTTTCATAAAAAG	HindIII
sepZ	F	CGCGGATCCGAAGCAGCAAATTTAAGTCCTTC	BamHI
	R	CCCAAGCTTTTAGGCATATTTCATCGCTAATGCAC	HindIII
orf12	F	CGCGGATCCAATCTTTTAGTTAAAAGAAACGTTG	BamHI
	R	CCCAAGCTTTCATGATGTCATCCTGCGAACG	HindIII
escN	F	CGCGGATCCATTTCAGAGCATGATTCTGTATTG	BamHI
	R	CGCGGATCCATTTCAGAGCATGATTCTGTATTG	PstI
orf15	F	CGCGGATCCTTGGACAGAATTTTATCTATTCGT	BamHI
	R	CCCAAGCTTCTAGTCAAAGTAATGTTCCTTTATGGC	HindIII
orf16	F	CGCGGATCCGCTTCTTTATGGAAGAGATTGTTTTACTCCTCGGG	BamHI
	R	CCCAAGCTTTTAATTTTCATATTCAATTGTGAACTCAATGGC	HindIII
sepQ	F	CGCGGATCCAAGCCATTGAGTTCACAATTG	BamHI
	R	CCCAAGCTTTTAATCACATACTATGCTAACAG	HindIII
espH	F	CGCGGATCCTCGTTATCAGGAGCGGTATTCAAG	BamHI
	R	CCCAAGCTTTCATAATACGCTATAAGAGGAAGC	HindIII
cesF	F	CGCGGATCCAATGAGAAATTTCGCACAGACCTTG	BamHI
	R	CCCAAGCTTTCAAGGTAAAAAATCTGTAGGTCTGG	HindIII
map	F	CGGGGTACCTTTAGTCCAATGACAATGGCAGGC	KpnI
	R	CCCAAGCTTCTACAATCGGGTATCCTGTACATG	HindIII
tir	F	CGGGGTACCCCTATTGGTAATCTTGGTCATAATC	KpnI
	R	CCCAAGCTTTTAGACGAAACGATGGGATCCC	HindIII
cesT	F	CGCGGATCCTCATCAAGATCTGAACTTTTATTAG	BamHI
	R	CCCAAGCTTTTATCTTCCGGCGTAATAATG	HindIII
escD	F	CGCGGATCCTTATCCTCATATAAAATAAAAC	BamHI

	R	CGCGGATCCTTATCCTCATATAAAATAAAAC	HindIII
sepL	F	CGCGGATCCGCTAATGGTATTGAATTTAATC	BamHI
	R	AAACTGCAGTCAAATAATTTCCTCCTTATAGTCG	PstI
espA	F	CGCGGATCCGATACATCAAATGCAACATCCGTTG	BamHI
	R	AAACTGCAGTTATTTACCAAGGGATATTGCTG	PstI
espD	F	CGCGGATCCCTTAACGTAAATAACGATACCCTG	BamHI
	R	CGGGGTACCTTAAATTCGGCCACTAACAATACG	KpnI
espB	F	CGCGGATCCAATACTATTGATAATACTCAAGTAACGATGG	BamHI
	R	AAACTGCAGTTACCCAGCTAAGCGACCCGATTGCCCC	PstI
cesD2	F	CGCGGATCCGTCGATACGTTTAATGATGAAGTG	BamHI
	R	AAACTGCAGTTAACTATTTACGTTCATTACGAACC	PstI
escF	F	CGCGGATCCAATTTATCTGAAATTACTCAAC	BamHI
	R	CCCAAGCTTTTAAAAACTACGGTTAGAAATGG	HindIII
orf29	F	CGCGGATCCGTTAATGATATTTCTGCTAATAAGATACTGG	BamHI
	R	AAACTGCAGTTAAAATCCTCGTACCCAGCCACTACC	PstI
espF	F	CGCGGATCCCTTAATGGAATTAGTAACGCTGC	BamHI
	R	CCCAAGCTTTTACCCTTTCTTCGATTGCTCATAGG	HindIII
*rorf1**	F	CGCGGATCCCCTCACCTCAAGAACACTCACTTTC	BamHI
	R	ACGCGTCGACTTACTTATTAGGGACAAATTTC	SalI
espG	F	CGCGGATCCATACTTGTTGCCAAATTGTTC	BamHI
	R	AAACTGCAGTTAAGTGTTTTGTAAGTACGTTTCAGATGCGG	HindIII
Non-LEE genes			
nleA	F	GGAAGATCTAACATTCAACCGACCATACAATC	BglII
	R	TCCCCCCGGGTTAGACTCTTGTTTCTTGG	XmaI
nleB	F	CGCGGATCCTTATCTTCATTAAATGTCCTTCAATCCAGC	BamHI
	R	CCCAAGCTTTTACCATGAACTGCAGGTATACATACTG	HindIII
nleB-1	F	CGCGGATCCCTTTCACCGATAAGGACAACTTTC	BamHI
	R	CGGGGTACCTTACCATGAACTGCATGTATACTG	KpnI
nleC	F	CGCGGATCCAAAATTCCCTCATTACAGTCCAAC	BamHI

	R	CCCAAGCTTTCATTGCTGATTGTGTTTGTCCAC	HindIII
nleD	F	CGCGGATCCCGCCCTACGTCCCTCAACTTGGTATTAC	BamHI
	R	CCCAAGCTTCTAAAGCAATGGATGCAGTCTTACCTG	HindIII
nleE	F	CGCGGATCCATTAATCCTGTTACTAATACTCAGGGCGTGTCCCCTATAAATACTAAATATGCTGAACAT	BamHI
	R	CCCAAGCTTCTACTCAATTTTAGAAAGTTTATTATTTATGTATTTCATATAACTGTCTATTTCCCCAGGC	HindIII
nleF	F	CGCGGATCCTTACCAACAAGTGGTTCTTCAGC	BamHI
	R	CCCAAGCTTTCATCCACATTGTAAAGATCCTTTG	HindIII
nleG	F	CGCGGATCCCCTGTCATATTAAACTTTTCGAGTG	BamHI
	R	CCCAAGCTTTCAAATTCTAGTGCATATATTTTGTGTGGC	HindIII
nleH1-1	F	CGCGGATCCTTATCGCCCTCTTCTATAAATTTGGGATGTTCATGG	BamHI
	R	CCCAAGCTTTTATATCTTACTTAATACTACACTAATAAGATCCAGC	HindIII
nleI	F	CGCGGATCCCAGGTTCTTCGTGCTCAAATGG	BamHI
	R	CCCAAGCTTTCATAAATACATTGTTCTTGAC	HindIII
nleG2-1	F	CGCGGATCCAATGTCCTTCGAGCTCAAGTAGCATCTAG	BamHI
	R	CCCAAGCTTTTAACTATCTTTTATAATGAAGTTTCCC	HindIII
nleG2-2	F	CGCGGATCCCCATTAACCTCAGATATTAGATCAC	BamHI
	R	CCCAAGCTTTCAATTACCCTTTATAACGAAGTTTCC	HindIII
nleG3	F	CGCGGATCCGTAATGCCTGGATTAGTATC	BamHI
	R	CCCAAGCTTTTAATGCAATTGAAATAAATAAG	HindIII
nleG5-1	F	CGCGGATCCCCTGTAGATTTAACGCCTTATATTTTACCTGGG	BamHI
	R	CCCAAGCTTTTAATTTTTTAAAACGAAGTTACCTCTGTCAGGG	HindIII
nleG6-1	F	CGCGGATCCCCTGTTACCACCTTAAGTATCCC	BamHI
	R	CGGGGTACCTCACTTACAACAAAAAGCTTCTC	KpnI
nleG8-2	F	CGCGGATCCCCAGTCATATTAAATTTTTCTAATGGAAGTG	BamHI
	R	CCCAAGCTTTTAAATACTGTTTTGTTGAAGTGGGTATATG	HindIII
nleG9	F	CGCGGATCCGACGCTTTTATTGTAGATCCTGTTC	BamHI
	R	CCCAAGCTTCTACACTGAATAACAATCACTCC	HindIII
espK	F	CGCGGATCCATGCTTCCTACATCGCAATTACGAC	BamHI
	R	CCCAAGCTTTTAAGAATATTTATATGTGGAACCAGAG	HindIII
espL2	F	CGGATCCCCAATAATAAACAAATCGGCATCAAATTATG	BamHI

	R	CCCAAGCTTTCAATTGGAATAATAATTATATACATCGAGG	HindIII
espM2	F	CGCGGATCCCCGATGAATACTACAGGTATGTC	BamHI
	R	CCCAAGCTTTCATCCCTGTATAGCACGCATC	HindIII
espR1	F	CGCGGATCCAAATTCCCTTCAATATTTAACAAAATAAAACC	BamHI
	R	CGGGGTACCTTAGTGATAAAAAGGCCATGAGCTGGAGG	KpnI
tccp	F	CGCGGATCCATTAACAATGTTTCTTCACTTTTTCC	BamHI
	R	CCCAAGCTTTCACGAGCGCTTAGATGTATTAATG	HindIII
espV	F	CGCGGATCCAGCGGAACCTCAGGTTCCTCG	BamHI
	R	CCCAAGCTTTCACAAAAAAGATTGGGGAGG	HindIII
espW	F	CGCGGATCCCCCAAAATATCATCAGTTGTATCATC	BamHI
	R	CCCAAGCTTTTAATTTCTAACCAAGGGGTCCCATG	HindIII
espX2	F	CGCGGATCCGATTGTTCAAAATGCAATGGTTATG	BamHI
	R	CCCAAGCTTTTACAGCCATGCGTCTGGCGTCCAC	HindIII
espX7	F	CGCGGATCCAAACATATAGAAGGTTCCTTTCCTG	BamHI
	R	CGGGGTACCTCAACGCCACGCAACAGGATAATAC	KpnI
espY1	F	CGCGGATCCAAAGTATCAGTTCCAGGCATGC	BamHI
	R	CCCAAGCTTTCATTCAATAATTGCGTTGTCAG	HindIII
espY2	F	CGCGGATCCAAAGTAAGAAACCCAGAACAGATTAG	BamHI
	R	CCCAAGCTTTCAGTCATACCAACGGCTATTGTTCG	HindIII
espY3	F	CGCGGATCCATGAAAACCATCACCAAACAACCG	BamHI
	R	CCCAAGCTTTCAGTCGACGAACTCATAATAATTGCTC	HindIII

6.2.4 Expression and purification of GST fusion proteins

Glutathione S-transferase fusion proteins were expressed and purified as described (Johnson, Osheim et al. 2004). Briefly, 500 mL LB + ampicillin (100 µg/mL) + chloramphenicol (50 µg/mL) was inoculated with 3 mL of an overnight culture containing the desired plasmids in BL21 cells. Bacteria were grown at 37ºC with shaking to an absorbance of 0.2 at 600 nm, where IPTG was added at a concentration of 0.25 mM and cultures were incubated for an additional 3 hours at 30ºC. Bacteria were sedimented by centrifugation, and resuspended in binding buffer (540 mM NaCl, 2.7 mM KCl, 10.15 mM Na_2HPO_4, 1.75 mM KH_2PO_4, 10 mM $MgCl_2$, 1% [vol/vol] Triton X-100, 50 µg of DNase I, 30 µg/mL PMSF, 1 µg/mL aprotinin 1 µg/mL pepstatin A, 10 µg/mL leupeptin [pH 7.4), followed by sonication (3 x 30 seconds with 1 second pulse, 12 mm probe with max power, Vibra-Cell, Sonics & Materials Inc, Dansbury, USA). Glutathione S-transferase fused proteins were purified by adding 1 mL of 1:1 slurry of Glutathione Sepahrose 4B beads (Amersham) in PBS to 10 mL of cleared lysate. The beads were then washed four times with 15 mL of binding buffer. The purity of proteins was assessed following SDS-PAGE using Coomassie Brilliant Blue staining (Laemmli 1970).

6.2.5 SDS-PAGE and Western blot analysis

Proteins were separated by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue (Laemmli 1970). Proteins were transferred to nitrocellulose membranes by electroblotting as described by the manufacturer (BioRad Laboratories). Western blot analysis was carried out using rabbit anti-T3SPs, STEC (O157, O26, O111 & O103) antibodies, anti-6x HIS monoclonal antibody (Becton, Dickinson) and bovine anti-STEC O157 polyclonal antibodies as described (Laemmli 1970; Li, Frey et al. 2000).

6.2.6 Production of Type III –secreted proteins and rabbit anti-T3SP polyclonal antibodies

Type III-secreted proteins from all STEC serotypes were prepared as described (Li, Frey et al. 2000). Briefly, overnight cultures of STEC serotypes were diluted 100-fold in M9 minimal media containing of 0.4% glucose, 0.1% Casamino acids, 44 mM

$NaHCO_2$, 8 mM $MgSO_4$ and 45 mM $KHCO_3$. Cultures were then incubated without shaking at 37ºC in a 5% CO_2 environment to an OD_{600} of 0.6-0.8. Bacteria was pelleted by centrifugation and the supernatant proteins concentrated by precipitation with 10% trichloroacetic acid. One mL quantities containing 50 μg of STEC T3SPs, and 30% Emulsigen®- D (MVP laboratories) were used to immunize female New Zealand white rabbits subcutaneously. The animals received two boosts, three weeks apart before being euthanized. Rabbits were bled according to the guidelines provided by the University Council on Animal Care (UCAC), under protocol number 1994-213. The collected blood was centrifuged and the serum was stored at -20ºC until further use.

6.2.7 Experimentally infected cattle

Two year old cattle were obtained from farms in Saskatchewan and housed at the University of Saskatchewan. Both animals were screened prior to challenge for shedding and existing titers against T3SPs from STEC O157:H7. Cattle received two oral challenges, 21 days apart of 500 mL containing 10^9 CFU/mL of STEC O157:H7. Serological conversion was measured by ELISA and Western blots.

6.2.8 Enzyme-Linked ImmunoSorbent Assay

Enzyme-Linked ImmunoSorbent Assays using rabbit anti-T3SPs serotypes STEC (O157, O26, O111 & O103), polyclonal antibodies against 6x HIS-tagged LEE and non-LEE proteins were completed as described (Li, Frey et al. 2000). The preimmune sera used was collected from a naïve animal with no previous history of diarrhea or previous contact with STEC serotypes. Briefly, plates (Immulon 2 HB, Thermo) were coated with antigen overnight at 4ºC. Plates were blocked for 1 hour with 1% nonfat dried milk (CO-OP, Canada) in Tris-Buffered Saline pH 7.6 with the addition of 0.05% Tween-20 (Sigma) (TBST). Serial dilutions were completed for primary antibody and incubated for 2 hours at room temperature. After washes, phosphatise-labelled labelled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) at a dilution of 1/2000 in TBST was incubated for 1 hour at room temperature, and the alkaline phosphate activity was detected using *p*-nitrophenyl phosphate (PNPP) (Sigma). The optical density was measured at 405nm

using a BioRad model 1350 microplate reader. Data are reported as median values and their ranges (Table 6.2 and Table 6.3).

For human sera, single-well dilutions were completed for each protein (in duplicate). Duplicate wells using naïve human sera were calculated to measure the background of each protein. The graphed ELISA OD value was measured by subtracting the naive value (from uninfected individuals) from the HUS-positive human sera. Duplicate values were averaged and three standard deviations calculated before subtraction. This subtraction method was selected over the traditional titer presentation due to limited volumes of HUS-positive human sera. The subtraction method was also used with the experimentally infected cattle sera in order to compare results with HUS-positive human sera.

6.2.9 Sera from human HUS patients

Sera were obtained from six HUS patients, who developed HUS as a result of STEC O157:H7 infections. All serum samples were collected roughly one week after the onset of clinical signs of disease. Naïve sera were collected from individuals with no history of recent diarrhea.

6.2.10 Challenge of mice with STEC O157 following immunization with two combinations of T3SPs

Four groups of 10 Balb/c mice were vaccinated with 0.5 μg of different combinations of T3SPs per mouse (Table 6.4). Group 1 received PBS (placebo group), Group 2 was vaccinated with STEC O157:H7 T3SPs + 30% Emulsigen®- D, Group 3 vaccinated 5 STEC recombinant proteins (EspG, NleH2-1, NleA, Chimeric Tir, and EspA) + 30% Emulsigen®- D and Group 4 with (EspG, NleH2-1, NleA, Chimeric Tir, EspRI, EspF, EspB,
EspD and EspA) + 30% Emulsigen®- D. All groups received two vaccinations 21 days apart. Fourteen days following the last vaccination all mice were challenged orally with 100μL of 10^9 CFU per mL of Nal^r *E. coli* O157 EDL933 strain in 20% sucrose. Two days prior to challenge, water was treated with 5 g/L of streptomycin to remove intestinal flora as described (Babiuk, Asper et al. 2008). Mice were also deprived of food

Table 6.2. ELISAs using anti-Type III secreted protein *E. coli* O157:H7 and non-O157 sera against recombinant STEC O157 LEE proteins. All proteins which had a positive reaction in the Western blots using rabbit anti-T3SPs *E. coli* O157, anti-T3SPs *E. coli* O26 anti-T3SPs *E. coli* O103 and anti-T3SPs *E. coli* O111 sera, were also tested with the same sera in ELISAs for quantitative measure. A total of 20 proteins were tested including 18 proteins which were positive in Western blots (summarized on Table 2) and 2 negative proteins (NleG6-1 and Map). Preimmune = preimmune sera; O157 = rabbit anti-O157 T3SPs polyclonal antibodies; O26 = rabbit anti-O26 T3SPs polyclonal antibodies; O103 = rabbit anti-O103 T3SPs polyclonal antibodies; O111 = rabbit anti-O111 T3SPs polyclonal antibodies. Values present ELISA titers. Responses were grouped statistically [A, B, C, D, E; differences in reactivity were examined using one-way ANOVA ($P<0.05$)]. All proteins were expressed and purified from STEC STEC O157:H7. The different letters (A, B, C, D, and E) represent statistical differences among the serum from the different serotypes tested against a single protein. Example, if the letters are the same for two serum tested, then it means that there is no statistical different between the two samples against the particular protein.

Protein	Preimmune	O157	O26	O103	O111
EscC	1525 (A)	6684 (B)	22390 (C)	7304 (B)	17222 (D)
SepD	265 (A)	6453 (B)	760 (C)	197773 (D)	1381 (C)
Tir	419 (A)	567692 (B)	522874 (C)	130202 (D)	489475 (C)
EspA	234 (A)	637500 (B)	297646 (C)	299648 (C)	395028 (B)(C)
EspD	224 (A)	412851 (B)	5694 (A)	384520 (B)	288509 (B)
EspB	179 (A)	511393 (B)	99719 (C)	474865 (B)	497104 (B)
EspF	332 (A)	937 (B)	6995 (C)	25672 (D)	86078 (E)
EspG	398 (A)	386863 (B)	5643 (C)	1123 (D)	422629 (B)
NleA	128 (A)	460507 (B)	63889 (C)	55801 (D)	20062 (D)
NleE	1177 (A)	6345 (B)	1224 (A)	2443 (C)	5714 (D)
NleF	313 (A)	1362 (B)	392 (A)	512229 (C)	4155 (D)
EspRI	3225 (A)	156521 (B)	6543 (C)	4407 (A)	6891 (C)
NleH	4283 (A)	24373 (B)	5997 (C)	19138 (D)	2919 (E)
NleI	413 (A)	5738 (B)	1757 (C)	19969 (D)	5310 (E)
NleG2-1	388 (A)	4566 (B)(C)	2587 (C)	121235 (D)	6563 (B)
NleG2-2	953 (A)	6719 (B)	2574 (A)(B)	84860 (C)	7027 (B)
TccP	368 (A)	1447 (B)	132429 (C)	27261 (D)	6875 (E)
EspY1	1958 (A)	1874 (A)	7273 (B)	8455 (B)	6318 (B)
NleG6-1	395 (A)(C)	4386 (B)	1360 (A)(C)	1502 (B)(C)	1399 (C)
Map	381 (A)	4542 (B)	469 (C)	1271 (D)	1530 (E)

Table 6.3 ELISAs median data using anti-Type III secreted protein *E. coli* O157:H7 and non-O157 sera against recombinant STEC O157 LEE proteins including minimum and maximum ranges. Preimmune = preimmune sera; O157 = rabbit anti-O157 T3SPs polyclonal antibodies; O26 = rabbit anti-O26 T3SPs polyclonal antibodies; O103 = rabbit anti-O103 T3SPs polyclonal antibodies; O111 = rabbit anti-O111 T3SPs polyclonal antibodies. Values present ELISA titers. The minimum and maximum ranges are present in brackets.

	Preimmune		O157
Protein	**Median (minimum, maximum)**	**Protein**	**Median (minimum, maximum)**
EscC	1525 (1472, 1620)	**EscC**	6684 (6471, 7008)
SepD	265 (245, 284)	**SepD**	6453 (6197, 6709)
Tir	419 (404, 451)	**Tir**	567692 (559658, 582009)
EspA	234 (213, 254)	**EspA**	637500 (522683, 752317)
EspD	224 (221, 236)	**EspD**	412851 (306215, 512608)
EspB	179 (160, 198)	**EspB**	511393 (412605, 610181)
EspF	332 (320, 352)	**EspF**	937 (895, 1047)
EspG	398 (394, 401)	**EspG**	386863 (343485, 430241)
NleA	128 (125, 130)	**NleA**	460507 (450098, 470915)
NleE	1177 (1076, 1200)	**NleE**	6345 (6302, 6548)
NleF	313 (290, 337)	**NleF**	1362 (1320, 1404)
EspRI	3225 (1810, 3687)	**EspRI**	156521 (114068, 190076)
NleH	4283 (3764, 4508)	**NleH**	24373 (22145, 25682)
NleI	413 (381, 443)	**NleI**	5738 (5397, 5744)
NleG2-1	388 (381, 394)	**NleG2-1**	4566 (4200, 4932)
NleG2-2	953 (461, 1445)	**NleG2-2**	6719 (6346, 7092)
TccP	368 (358, 378)	**TccP**	1447 (1390, 1504)
EspY1	1958 (1859, 1961)	**EspY1**	1874 (1735, 1893)
NleG6	395 (394, 1233)	**NleG6**	4386 (4349, 4602)
Map	381 (372, 401)	**Map**	4542 (4508, 4801)

	O26		O103
Protein	**Median (minimum, maximum)**	**Protein**	**Median (minimum, maximum)**
EscC	22390 (21205, 24306)	**EscC**	7304 (6621, 7436)
SepD	760 (428, 1092)	**SepD**	197773 (163840, 231705)

Tir	522874 (499472, 528599)	**Tir**	130202 (102125, 121999)
EspA	297646 (260080, 335212)	**EspA**	299648 (205319, 393977)
EspD	5694 (5653, 5879)	**EspD**	384520 (231705, 537615)
EspB	99719 (99200, 100238)	**EspB**	474865 (472049, 477681)
EspF	6995 (6850, 7110)	**EspF**	25672 (24581, 40120)
EspG	5643 (5394, 5891)	**EspG**	1123 (1074, 1171)
NleA	63889 (5615, 7162)	**NleA**	55801 (25169, 86432)
NleE	1224 (957, 1546)	**NleE**	2443 (1805, 2778)
NleF	392 (376, 408)	**NleF**	512229 (475930, 548527)
EspRI	6543 (6253, 6859)	**EspRI**	4407 (1731, 4649)
NleH	5997 (5714, 9078)	**NleH**	19138 (17513, 19431)
NleI	1757 (1662, 3378)	**NleI**	19969 (18922, 30433)
NleG2-1	2587 (1487, 3687)	**NleG2-1**	121235 (99200, 143270)
NleG2-2	2574 (1568, 3579)	**NleG2-2**	84860 (76006, 93713)
TccP	132429 (101018, 163840)	**TccP**	27261 (26488, 28033)
EspY1	7273 (7239, 7593)	**EspY1**	8455 (2703, 5823)
NleG6	1350 (1171, 1549)	**NleG6**	1502 (1340, 1587)
Map	469 (457, 484)	**Map**	1271 (1177, 1358)

	O111
Protein	**Median (minimum, maximum)**
EscC	17222 (15864, 17924)
SepD	1381 (1346, 1415)
Tir	489475 (463410, 537615)
EspA	395028 (384477, 405578)
EspD	288509 (196625, 307269)
EspB	497104 (475930, 518278)
EspF	86078 (68886, 98494)
EspG	422629 (388984, 456274)
NleA	20062 (18357, 21767)
NleE	5714 (5397, 5832)
NleF	4155 (3579, 4731)
EspRI	6891 (5757, 7585)
NleH	2919 (1563, 3310)
NleI	5310 (4964, 5399)

NleG2-1	6563 (6145, 6981)
NleG2-2	7027 (7022, 7031)
TccP	6875 (6828, 6922)
EspY1	6318 (6282, 6878)
NleG6	1399 (1336, 1493)
Map	1530 (1504, 1697)

Table 6.4 Vaccination groups used in the challenge of mice with STEC O157 following immunization. Four groups of 10 Balb/c mice were vaccinated with different combinations of T3SPs.

Group 1	0.1 M PBS (Placebo)
Group 2	STEC O157:H7 T3SPs + 30% Emulsigen®- D
Group 3	EspG, NleH2-1, NleA, Chimeric Tir, and EspA + 30% Emulsigen®- D
Group 4	EspG, NleH2-1, NleA, Chimeric Tir, EspRI, EspF, EspB, EspD and EspA + 30% Emulsigen®- D

and water 18 hours prior to challenge. Fecal samples were collected every 2 days for 2 weeks to investigate STEC shedding following vaccinations.

On average, 0.1 gram of fecal matter was collected from each mouse (3-4 pellets) and placed in an Eppendorf tube. One milliliter of LB broth was added to each tube which was incubated at room temperature for 3 hours to allow the pellet to soften. The tubes were then centrifuged to allow the dispersal of pellets. Samples were diluted in PBS to 10^{-6} and plated on CT-SMAC agar plates (Mackonkey agar + cefiximine 0.05 mg/L + tellurite 2.5 mg/L + nalidixic acid 15 mg/L). Plates were then incubated overnight at 37 ºC. The next morning colonies were counted and *E. coli* O157 confirmed by latex agglutination tests (Dry S-SPOT, Oxoid, UK).

6.2.11 Statistical analysis

Differences in antisera reactivity with each protein were examined using one-way analysis of variance (ANOVA). When data were normally distributed, the ANOVA was performed on the raw data. Non-normally distributed data were either rank or log transformed and the ANOVA was then performed on the data. Means of raw or transformed data were compared using the Least Significant Difference method. Differences were considered significant when $P<0.05$.

6.3 Results

6.3.1 Western blot analysis of bovine and rabbit sera specific to STEC O157:H7 and non-O157 T3SPs against recombinant purified STEC O157:H7 secreted proteins

Bovine and rabbit sera raised against STEC O157:H7 T3SPs were tested against 37 LEE purified proteins to investigate if the pattern of reactive proteins was similar. Both rabbit and bovine sera reacted with the same proteins (Tir, EspA, EspB, EspG and EspD) and the only difference observed was in the band intensity (Figure 6.1B and 6.1G). Rabbit O26-, O103-, O111- and O157-specific sera raised against T3SPs were also tested against the 37 LEE proteins to determine which cross-reactive proteins were present in the bacterial culture supernatant and to investigate the cross-reactive properties of the proteins with non-O157 sera (Figure 6.1 A-E and summarized on Table 6.5). The pattern of recognized LEE proteins was comparable, where Tir, EspA, EspB, and EspD reacted with sera from all serotypes. The EspF and EspG protein were detected by the majority of the sera and SepD and EscC were detected by at least one individual serum tested.

A total of 29 non-LEE purified STEC O157:H7 secreted proteins were also tested against rabbit O26-, O103-, O111- and O157-specific sera (Figure 6.2 and 6.3 and summarized on Table 6.5). This pattern of recognized proteins varied, where only NleA and TccP reacted with all sera, NleE and NleH with the majority, and EspY1, NleG2-1, NleG2-2, NleI, EspR1 and NleF with at least one individual serum tested. Overall, O157-specific serum reacted to the least number of purified proteins (8 of 66) while O103-specific serum reacted to the most proteins (15 of 66). Rabbit preimmune serum was used as a negative control against all proteins (Figure 6.1F, Figure 6.2F and 6.3F), as well as an anti-6x HIS monoclonal antibody to ensure that the correct protein was purified (Figure 6.1H, Figure 6.2H and 3H). The Tir protein, which was not HIS-tagged, was supplied by Bioniche Life Sciences.

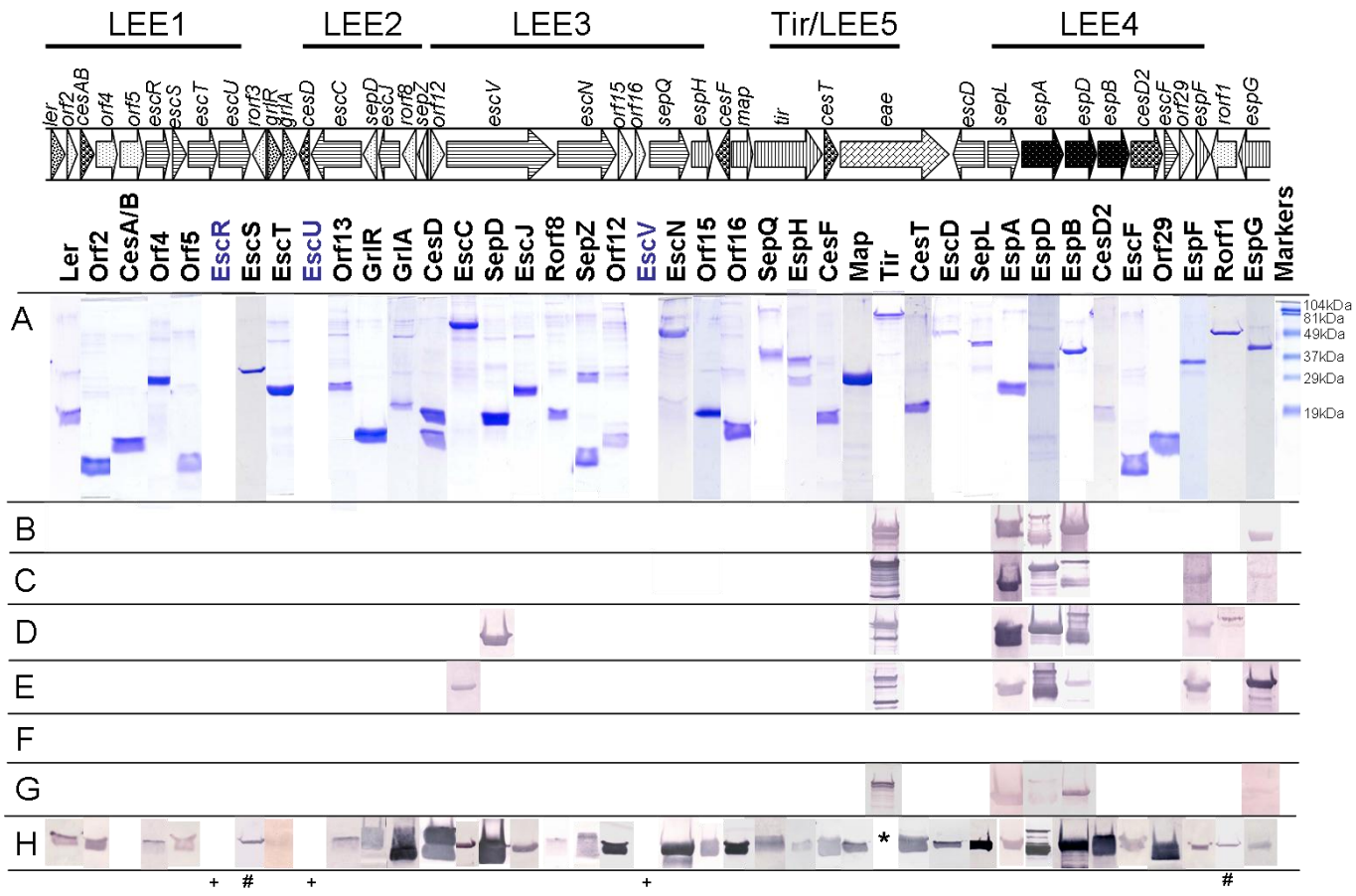

LEE1
LEE2
LEE3
Tir/LEE5
LEE4
ler orf2 cesAB orf4 orf5 escR escS escT escU rorf3 grlR grlA cesD escC sepD escJ rorf8 sepZ orf12 escV escN orf15 orf16 sepQ espH cesF map tir cesT eae escD sepL espA espD espB cesD2 escF orf29 espF rorf1 espG
Ler Orf2 CesA/B Orf4 Orf5 EscR EscS EscT EscU Orf13 GrlR GrlA CesD EscC SepD EscJ Rorf8 SepZ Orf12 EscV EscN Orf15 Orf16 SepQ EspH CesF Map Tir CesT EscD SepL EspA EspD EspB CesD2 EscF Orf29 EspF Rorf1 EspG Markers
104kDa
81kDa
49kDa
37kDa
29kDa
19kDa
A
B
C
D
E
F
G
H
*
+
#
+
+
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Figure 6.1. Western blots using anti- Type III secreted protein *E. coli* O157:H7 and non-O157 sera against recombinant STEC O57 LEE proteins. In total 40 LEE genes were selected for over expression and purification. All genes were cloned and sequenced using the Qiagen pQE-30 HIS-tagged vector cloning system (except EscS and Rorf1 cloned into the pGEX-5X-1 for GST fusion purification). Thirty seven proteins were purified and tested against sera. A) Proteins visualized by SDS-PAGE using Coomassie Brilliant Blue. B) Western blots using rabbit anti-T3SPs *E. coli* O157. C) Western blots using rabbit anti-T3SPs *E. coli* O26. D) Western blots using rabbit anti-T3SPs *E. coli* O103. E) Western blots using rabbit anti-T3SPs *E. coli* O111. F) Western blots using rabbit preimmune sera. G) Western blots using bovine anti-T3SPs *E. coli* O157. H) Western blots using anti-6x HIS monoclonal antibody. # = GST fused proteins; + = Membrane proteins not purified. * = Tir protein supplied by Bioniche Life Sciences. *Orf* unknown function, chaperone, intimin, secreted protein, regulator, translocator protein, structural protein.

Table 6.5. Summary of reactive recombinant STEC O157 Type III secreted proteins against rabbit O26-, O103-, O111- and O157-specific sera, and sera from O157-experimentally infected and O157-vaccinated cattle. A) Table represents LEE and non-LEE proteins which reacted against O26-, O103-, O111- and O157-specific sera in Western blots. O157 = rabbit anti-O157 T3SPs polyclonal antibodies; O26 = rabbit anti-O26 T3SPs polyclonal antibodies; O103 = rabbit anti-O103 T3SPs polyclonal antibodies; O111 = rabbit anti-O111 T3SPs polyclonal antibodies. B) Table represents LEE and non-LEE proteins which reacted against sera from O157-experimentally infected and O157-vaccinated cattle. + = represent reactivity.

A)

	Proteins	O157	O26	O103	O111
LEE	EscC				+
	SepD			++	
	Tir	++	++	+++	++
	EspA	+++	+++	+++	++
	EspD	+++	++	+++	+++
	EspB	+++	++	+++	++
	EspF		++	++	++
	EspG	++	+		+++
Non-LEE	NleA	+++	++	+++	+++
	NleE			+	+
	NleF			++	
	EspR1	++			
	NleH		+	++	+
	NleI			++	
	NleG2-1			++	
	NleG2-2			+	
	TccP	+	++	+++	+
	EspY1			+	++

B)

	Proteins	Vaccinated with STEC O157:H7 TTSPs	Experimentally infected with STEC O157:H7
LEE	Tir	++	+
	EspA	++	+
	EspD	+	++
	EspB	++	+++
	EspG	+	
Non-LEE	EspM2		+
	NleA	+	+
	TccP		+

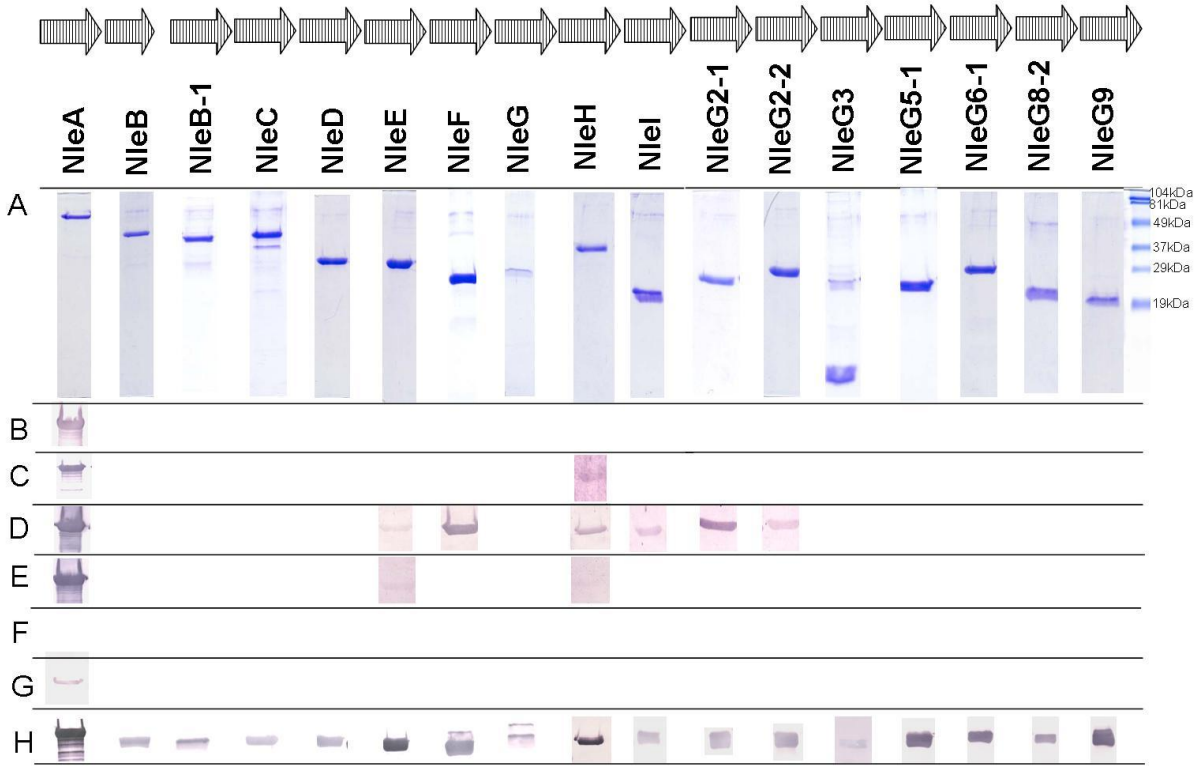

Figure 6.2. Western blots using anti- Type III secreted protein *E. coli* O157:H7 and non-O157 sera against recombinant STEC O57 LEE proteins. Seventeen Nle genes were selected for over -expression and purification. All genes were cloned and sequenced using the Qiagen pQE-30 HIS-tagged vector cloning system. Seventeen proteins were purified and tested against sera. A) Proteins separated by SDS-PAGE and visualized using Coomassie Brilliant Blue stain. B) Western blots using rabbit anti-T3SPs from *E. coli* O157. C) Western blots using rabbit anti-T3SPs from *E. coli* O26. D) Western blots using rabbit anti-T3SPs from *E. coli* O103. E) Western blots using rabbit anti-T3SPs from *E. coli* O111. F) Western blots using rabbit preimmune sera. G) Western blots using bovine anti-T3SPs *E. coli* O157. H) Western blots using anti-6x HIS monoclonal antibody. Secreted protein ▨.

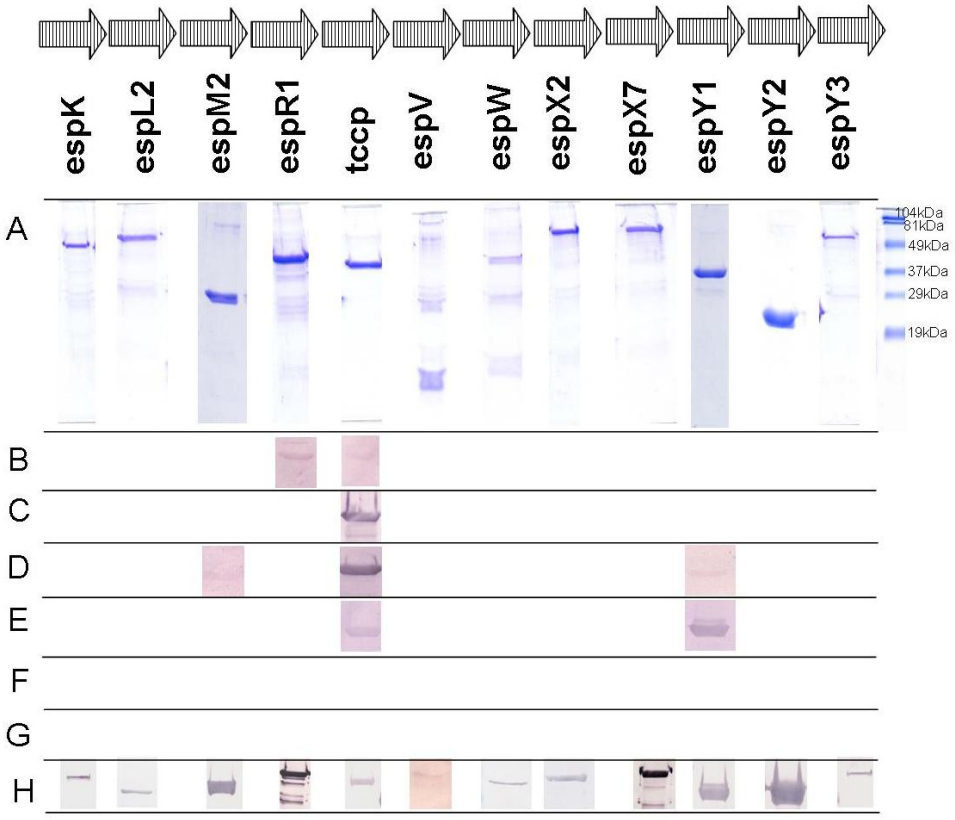

Figure 6.3. Western blots using anti- Type III secreted protein *E. coli* O157:H7 and non-O157 sera against recombinant STEC O57 LEE proteins. Twelve non-LEE genes were selected for over expression and purification. All genes were cloned and sequenced using the Qiagen pQE-30 HIS-tagged vector cloning system. Twelve proteins were purified and tested against sera. A) Proteins seperated by SDS-PAGE and visualized using Coomassie Brilliant Blue stain. B) Western blots using rabbit anti-T3SPs from *E. coli* O157. C) Western blots using rabbit anti-T3SPs E. coli O26. D) Western blots using rabbit anti-T3SPs *E. coli* O103. E) Western blots using rabbit anti-T3SPs *E. coli* O111. F) Western blots using rabbit preimmune sera. G) Western blots using bovine anti-T3SPs *E. coli* O157. H) Western blots using anti-6x His monoclonal antibody. Secreted protein .

6.3.2 A serological analysis by ELISA of rabbit sera raised to STEC O157:H7 and non-O157 T3SPs against recombinant purified STEC O157:H7 secreted proteins

To obtain a quantitative measure of the response against STEC T3SPs, ELISAs were used to determine the reactivity of purified STEC O157:H7 secreted proteins with rabbit sera raised to STEC O157, O26, O103 and O111 T3SPs. A total of 20 proteins were tested including 18 proteins which reacted positively in Western blots (summarized in Table 6.2) and 2 proteins (NleG6-1 and Map) that did not react. The majority of proteins gave similar findings when compared with the Western blot results described (Table 6.5). Antiserum which was reactive in Western blots gave ELISA titers that were significantly higher than both the preimmune and non-reactive sera. The two proteins used that did not react to any of the sera tested in Western blots showed low readings in ELISA analysis compared to the reactive proteins, even though some of the antisera was significantly different from the preimmune serum. However, a number of proteins which were positive in Western blots with O26-, O103-, O111- and O157-specific sera demonstrated mixed results using ELISA analysis (NleE, NleH and EspY1). Statistical differences were considered significant when $P<0.05$.

6.3.3 Western blot and ELISA analysis of sera from STEC O157:H7 experimentally infected cattle against recombinant purified STEC O157:H7 secreted proteins

Sera collected from cattle which had been experimentally infected with STEC O157:H7 was tested against the 66 purified STEC O157:H7 secreted proteins. Using Western blot analysis, 6 of these 66 proteins reacted against the sera from infected cattle (summarized on Table 6.5). Four of these 6 proteins also reacted with sera from cattle vaccinated with STEC O157:H7 T3SPs (Tir, EspA, EspB and EspD) (Figure 6.1G). In addition, sera from infected cattle also recognized TccP and EspM2 (Table 6.5), while vaccinated cattle recognized NleA and EspG (Figure 6.1G, appendix Figure 6.2G and summarized on Table 6.5). ELISA analysis was also completed using the 66 proteins against sera from experimentally infected cattle. A total of 7 proteins gave an ELISA OD_{600} value greater than 0.050 after the subtraction calculation from the preimmune control sera (Figure 6.4 and Figure 6.5). Five of the 7 proteins resulted in significant reactivity when

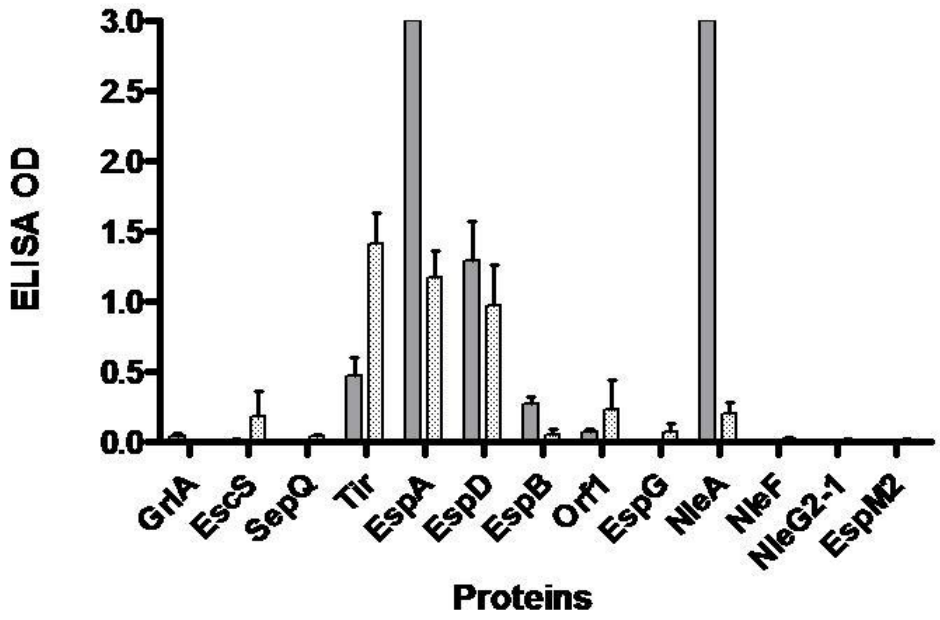

Figure 6.4. Antibody response of sera from STEC O157:H7 experimentally infected cattle and human sera from HUS patients against STEC O157 secreted proteins. Sixty six purified proteins were tested where only reactive proteins (ELISA OD higher than 0.050) were graphed. Negative proteins not shown on graph consist of: Ler, Orf2, CesA/B, Orf4, Orf5, EscT, Rorf13, GrlR, CesD, EscC, SepD, EscJ, Orf8, SepZ, Orf12, EscN, Orf16, EspH, CesF, Map, CesT, EscD, SepL, CesD2, EscF, Orf29, EspF, NleB, NleB2-1, NleC, NleE, NleG, NleH1-2, NleI, NleG2-2, NleG3, NleG5-1, NleG6-1, NleG8-2, NleG9, EspK, EspL2, EspR1, TccP, EspV,EspW, EspX2, EspX7, EspY1, EspY2 and EspY3. Single well dilutions of sera were used for each protein. Preimmune cattle serum was used to calculate background values against each protein. The graphed ELISA OD value represents the mean plus a standard deviation of samples (6 samples of human HUS patients and 2 samples from experimentally infected cattle**)** which were calculated by subtracting the preimmune value from the infected cattle value. Duplicate values were averaged and three standard deviations calculated before subtraction. Sera from experimentally infected cattle ■, sera from human from HUS patients ▒**.**

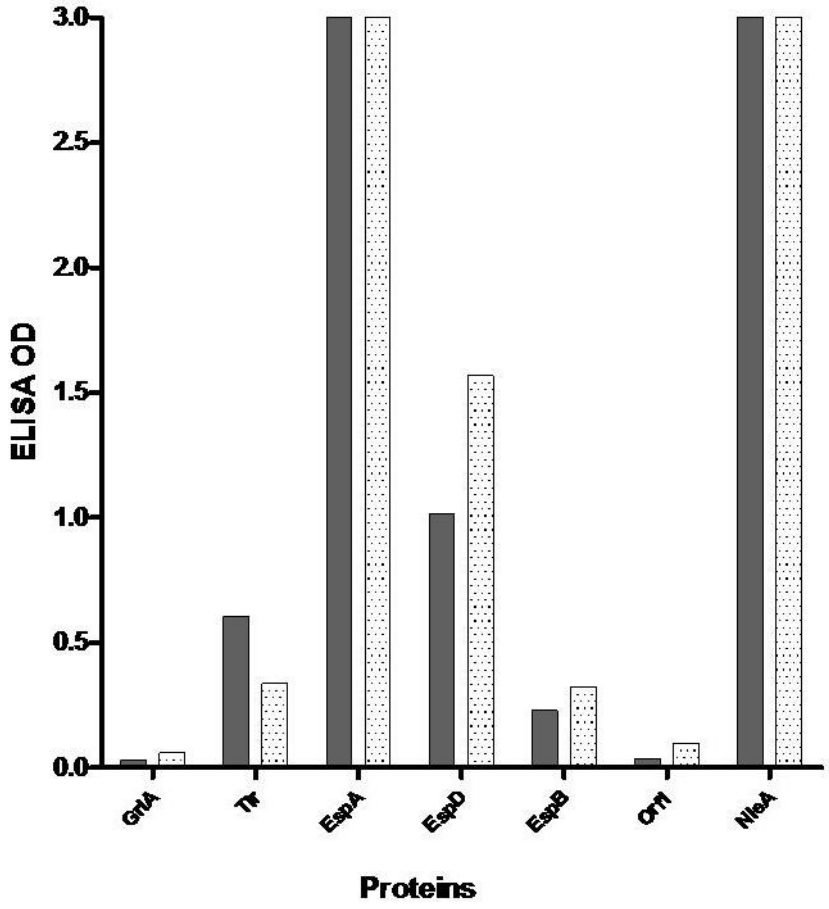

Figure 6.5. Antibody response of sera from STEC O157:H7 experimentally infected cattle against STEC O157 secreted proteins. Sixty six purified proteins were tested where only reactive proteins (ELISA OD higher than 0.050) were graphed. Negative proteins not shown on graph consist of: Ler, Orf2, CesA/B, Orf4, Orf5, EscS, EscT, Rorf13, GrlR, GrlA, CesD, EscC, SepD, EscJ, Orf8, SepZ, Orf12, EscN, Orf16, SepQ, EspH, CesF, Map, CesT, EscD, SepL, CesD2, EscF, Orf29, EspF, EspG, NleB, NleB2-1, NleC, NleE, NleF, NleG, NleH1-2, NleI, NleG2-1, NleG2-2, NleG3, NleG5-1, NleG6-1, NleG8-2, NleG9, EspK, EspL2, EspM2, EspR1, TccP, EspV, EspW, EspX2, EspX7, EspY1, EspY2 and EspY3. Single well dilutions of sera were used for each protein. Preimmune cattle serum was used to calculate background values against each protein. The graphed ELISA OD value was measured by subtracting the preimmune value from the infected cattle value. Duplicate values were averaged and three standard deviations calculated before subtraction. **Animal 1 ■, animal 2 ▨.**

either the sera from experimentally infected or vaccinated animals against T3SPs were used (Tir, EspA, EspD, EspB and NleA).

6.3.4 Reactivity of human sera from HUS patients against recombinant purified STEC O157:H7 secreted proteins

To determine the profile of T3SPs recognized by sera from HUS patients previously infected with STEC, we tested sera from six individuals against the 66 purified STEC O157:H7 secreted proteins. Twelve proteins gave an ELISA OD reading of over 0.050 after the subtraction of background values calculated from the naive sera. Four proteins (Tir, EspD, EspA and NleA) reacted with the majority of sera tested (Figure 6.4 and Figure 6.6), while EspG, EspB, Rorf1 and EscS demonstrated significant levels of reaction to at least one individual serum sample. The highest level of reactivity was seen with the Tir protein where all six samples demonstrated elevated readings.

6.3.5 Protective capacity of a recombinant Type III secreted proteins

Two different mixtures of secreted proteins (5 proteins and 9 proteins) were used to vaccinate mice and test the level of shedding following challenge with STEC O157. Both cocktails were also evaluated against a placebo group and the current Econiche STEC O157 T3SPs vaccine. A significant difference was observed in shedding overall and can be clearly seen from day 2 to 8 (Figure 6.7). After day 8 the shedding appears to plateau for all groups. The cocktail that contained 9 proteins (EspG, NleH2-1, NleA, Chimeric Tir, EspRI, EspF, EspB, EspD and EspA) reduced shedding to levels equivalent to the STEC O157 T3SP vaccine. On the other hand the cocktail that contained 5 proteins (EspG, NleH2-1, NleA, Chimeric Tir, and EspA) did not significantly reduce shedding when compared to the placebo. A significant difference was observed in shedding between the placebo group and the STEC O157 T3SPs group.

Statistical analysis of mouse fecal shedding was completed by incorporating repeated measures using the ANOVA test where the data was summed over time. The sums which were not normally distributed were log transformed and one-way ANOVA test used followed by Tukey's comparison of means test. Over the duration of the study

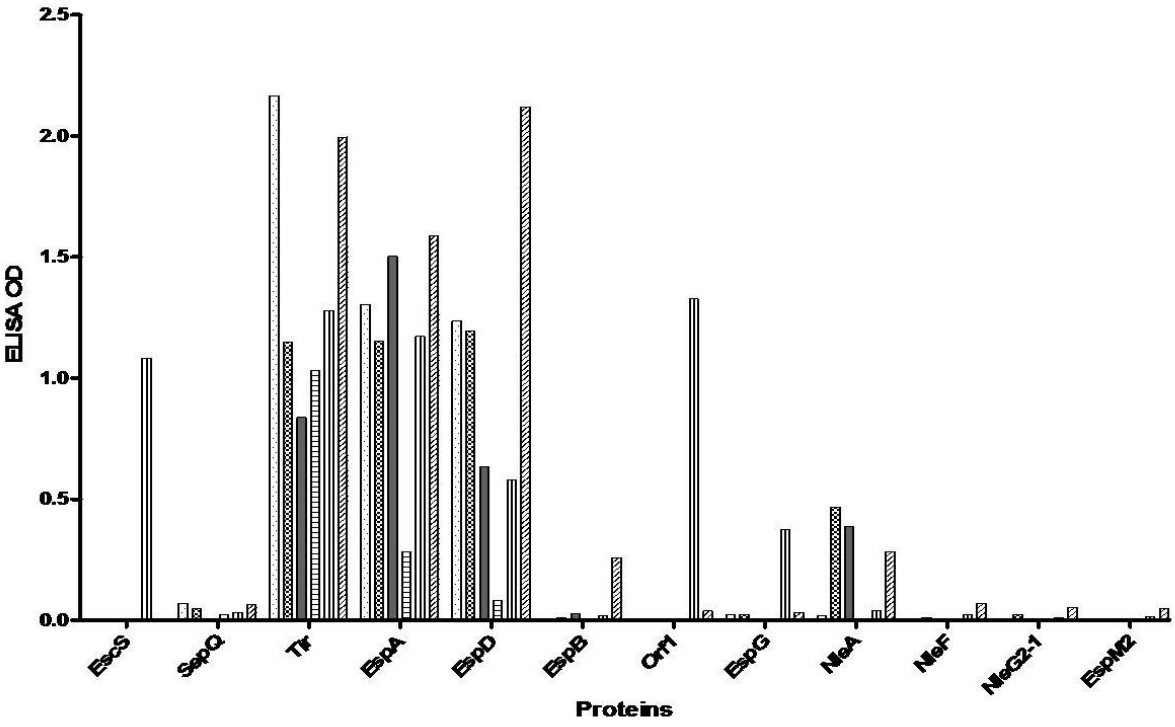

Figure 6.6. Antibody response of human sera from hemolytic-uremic syndrome patients against STEC O157 secreted proteins. Sixty six purified proteins were tested where only reactive proteins (ELISA OD higher than 0.050) were graphed. Negative proteins not shown on graph consist of: Ler, Orf2, CesA/B, Orf4, Orf5, EscT, Rorf13, GrlR, GrlA, CesD, EscC, SepD, EscJ, Orf8, SepZ, Orf12, EscN, Orf16, EspH, CesF, Map, CesT, EscD, SepL, CesD2, EscF, Orf29, EspF, NleB, NleB2-1, NleC, NleE, NleG, NleH1-2, NleI, NleG2-2, NleG3, NleG5-1, NleG6-1, NleG8-2, NleG9, EspK, EspL2, EspR1, TccP, EspV,EspW, EspX2, EspX7, EspY1, EspY2 and EspY3. Single well dilutions of human sera at 1:500 were used for each protein. Naïve human sera were calculated to measure the background of each protein. The graphed ELISA OD value was measured by subtracting the naive value from the HUS positive human sera. Duplicate values were averaged and three standard deviations calculated before subtraction. **Sample 1 , sample 2 , sample 3 , sample 4 , sample 5 , sample 6 .**

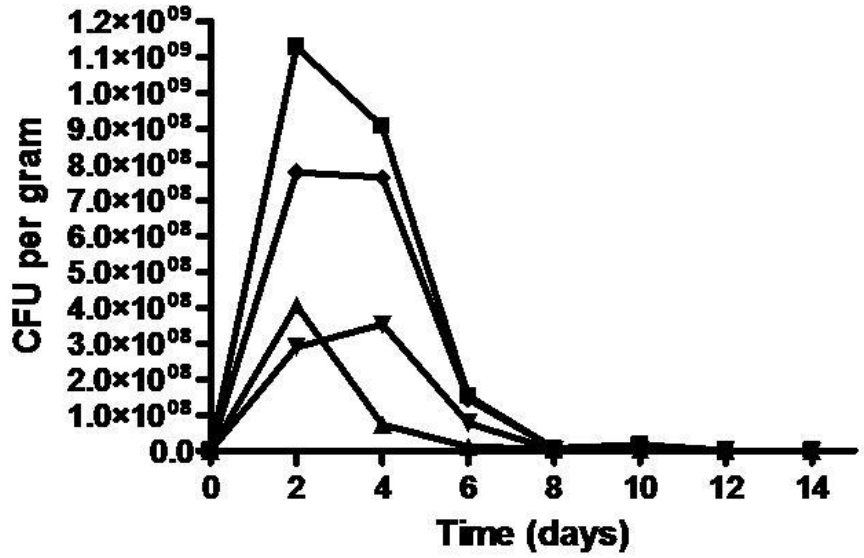

Figure 6.7. Challenge of mice with STEC O157 following immunization with two combinations of T3SPs. Four groups of 10 Balb/c mice were vaccinated with 0.5 µg of different combinations of T3SPs per mouse.(■) Groups 1 received PBS (placebo group); (▲) Group 2 was vaccinated with STEC O157:H7 T3SPs + 30% Emulsigen®- D; (♦) Group 3 vaccinated 5 STEC recombinant proteins (EspG, NleH2-1, NleA, Chimeric Tir, and EspA) + 30% Emulsigen®- D; (▼) Group 4 with (EspG, NleH2-1, NleA, Chimeric Tir, EspRI, EspF, EspB, EspD and EspA) + 30% Emulsigen®- D. All groups received two vaccinations 21 days apart. Two weeks following the last vaccination all mice were challenged with 100µL oral dose of 10^9 CFU per mL of Nal^r *E. coli* O157 strain in 20% sucrose. Two days prior to challenge, water was treated with 5 g/L of streptomycin to remove intestinal flora. Mice were also deprived of food and water 18 hours prior to challenge. Fecal samples were collected every 2 days for 2 weeks to investigate STEC shedding following vaccinations.

there were significant differences among the groups ($P<0.0001$). Both groups 2 and 3 were significantly different from group 1 and group 2.

6.4 Discussion

The production and secretion of T3SPs used for the colonization of a host are essential to the virulence of STEC O157:H7. These proteins are involved in the formation of A/E lesions which are critical for bacterial survival in both bovine and human hosts. Type III secreted proteins have been shown to have protective properties, as vaccination with a culture supernatant containing T3SPs significantly reduced the number of animals shedding STEC, as well as the number of STEC O157:H7 shed in individual fecal samples, after an experimental challenge (Potter, Klashinsky et al. 2004). At this time, only a small number of LEE and non-LEE secreted proteins have been identified as being present in the supernatant used for vaccination. Since the supernatant contains several proteins which are critical for protection, it would be beneficial to discover which specific secreted proteins are responsible for this effect. In this study, a total of 66 LEE and non-LEE proteins were studied to determine their immunogenicity and serological cross-reactivity, and to investigate their presence in the bacterial supernatant previously used to vaccinate against STEC O157:H7.

Initially we attempted to express and purify all genes found within the LEE Island (excluding intimin). We were however, unable to express and purify three structural proteins (EscR, EscU and EscV). These proteins are believed to be part of the inner membrane structure of the Type III apparatus and are not thought to be crucial in protection against infection.

The majority of LEE proteins that reacted with serum raised against STEC O157:H7 secreted proteins consisted of secreted effectors and structural proteins involved in the assembly of the external portion of the secretion apparatus. These results were expected since the majority of regulators, inner membrane and periplasmic structural proteins do not appear to be secreted but instead remain within the bacterium. The reaction of structural and regulatory proteins (SepD and EscC) with sera raised against T3SPs could be a result of bacterial lysis leading to contamination of the supernatant with these proteins.

Several LEE proteins such as Tir and EspA have been shown to be cross-reactive with sera raised against T3SPs from non-O157 serotypes (Asper, Sekirov et al. 2007). In

this study we have identified several other LEE and non-LEE proteins which are also cross-reactive with O26-, O103-, O111- and O157-specific sera. The initial cross-reactivity observed with Tir and EspA was accredited to their sequence homology within STEC serotypes. Although many of the STEC serotypes used in this study have not been fully sequenced, the observed cross-reactivity in this study could also be related to sequence homology.

The ELISA results presented in Table 6.2 were in agreement with the Western blot results for the majority of proteins which reacted against the STEC T3SP antisera (summarized in Table 6.5). Proteins which were identified as reactive by Western blotting gave ELISA readings that were significantly higher than the preimmune or a non-reactive serum. In general, for many of the proteins, non-reactive serum still gave an ELISA value that was significantly higher than the preimmune sera. However, this value when compared to a positive serum value for the same protein was still significantly less. The values acquired with the non-responsive sera could also be explained due to the level of sensitivity of the assays, where the sensitivity of ELISAs is much higher than a Western blot. A number of proteins gave mixed results. Proteins such as NleE which are positive on Western blots did not respond strongly on an ELISA. Interestingly, the reactivity on the Western blots was very weak, which could explain the mixed and small response on ELISA. Two other proteins, NleH and EspY1, also produced mixed results in ELISA values. In both cases, a Western blot based non-reactive serum, gave ELISA values equivalent to a reactive sera to the particular protein. These results could also be linked to the sensitivity of the assay and the level of protein denaturation which occurs between the two assays. Proteins, when used in a Western blot, undergo an SDS treatment that allows proteins to denature more than in an ELISA. Denaturation allows the exposure or the concealment of epitopes, which could explain the varying results.

The ELISA data in Table 6.2 also demonstrates how STEC strains can be grouped based on reactivity and secretion profiles. For example, proteins such as Tir, EspA, EspB and NleA are all secreted and appear to be cross-reactive with sera specific for all serotypes tested. Other proteins such as EspF, TccP, NleG2-1 and NleG2-2 are cross-reactive with a number of non-O157 serotypes such as STEC O103 but not with STEC O157 serum, as clearly observed by the low titers with the STEC O157 sera. The lack of

reactivity observed with STEC O157 sera could be a result of low secretion levels of the specific protein. However, further testing is required to confirm this hypothesis. For the majority of proteins which resulted in low titers with non-O157 sera, we are unable to conclude if these results are due to reduced homology, secretion levels, or the presence or absence of specific epitopes, since all proteins were expressed from STEC O157 genes. Interestingly, in chapter 5, the epitope mapping of the intimin binding domain of the Tir protein from STEC serotypes O157, O26, O103 and O111 has shown STEC O157 only possesses a single reactive epitope using homologous sera, while the non-O157 serotypes possess and react against multiple epitopes. However, in order to properly answer this question all proteins tested would have to also be purified from all non-O157 serotypes used.

The main reservoir for STEC is ruminants, and cattle are considered the most important source of human infection. These animals are colonized by highly virulent STEC strains without ever causing disease. Interestingly, STEC are still able to cause A/E lesions in cattle intestinal epithelia (Dean-Nystrom, Stoffregen et al. 2008). In humans, STEC infection involving A/E lesions leads to hemorrhagic colitis which results in complications such as HUS and TTP (Gyles 2007). In this study we compared sera from experimentally infected cattle and human HUS patients against LEE and non-LEE T3SPs to investigate the recognized pattern of purified proteins. In general, the majority of immunogenic proteins were recognized by both bovine and human sera. While the cattle response against the purified proteins was fairly consistent, the magnitude of the response by the human HUS sera appears to differ. However, the Tir protein gave the highest response of all tested proteins. Although Tir is one of the largest proteins that we purified the size does not correlate with the response, but instead is related to the immunogenicity of the protein. The bulk of positive immunogenic proteins appear to be structural components involved in the secretion of T3SPs. These results, in combination with the development of A/E lesions previously reported in humans and cattle, confirm that protein secretion is functional during natural infection of both hosts. In addition the above immunogenic proteins are found in genetic mobile elements which have been highlighted by Karmali and colleagues to play a role in the characterization of serotypes

into seropathotypes A through E, which are based on the occurrence in human disease, outbreaks and HUS cases (Karmali, Mascarenhas et al. 2003).

The majority of immunogenic proteins were LEE-based (Tir, EspB, EspD and EspA) and have been shown to participate in the colonization by STEC serotypes. Purified EspB, EspA and Tir have been previously tested with sera from HUS patients, where results also demonstrated that these proteins are recognized by a host during natural infection (Li, Frey et al. 2000). The only purified non-LEE protein that appears to be highly immunogenic is NleA. This protein has been previously shown to be involved in the modulation of virulence by the A/E pathogen, *Citrobacter rodentium* (Lee, Kelly et al. 2008). However, the role of NleA in the colonization of humans and cattle remains unknown.

In 2004 Potter *et al*, reported that the vaccination of cattle using secreted proteins of STEC O157 significantly reduced the numbers of bacteria shed in feces, the numbers of animals that shed, and the duration of shedding (Potter, Klashinsky et al. 2004). This vaccine has been recently tested in large scale commercial feedlots and the results have been promising. A large study involving 20,556 cattle in 19 different feedlots demonstrated that a two-dose vaccination reduced the prevalence of fecal shedding and the probability of environmental transmission of STEC O157 (Smith, Moxley et al. 2008). The result of another large feedlot trial using the same two-dose vaccination schedule showed that vaccination of the majority of cattle within a pen resulted in significant herd immunity following challenge with STEC O157 (Peterson, Klopfenstein et al. 2007).

A dilemma with the current T3SPs vaccine is the production cost and time necessary to produce large quantities of antigen. The optimal path would be the development of a second generation recombinant vaccine, which would simplify production and reduce costs. However, a second generation vaccine must be able to protect and reduce shedding as efficiently as, if not better than, the current version. Based on the identification of a number of immunogenic effector proteins, we have developed a vaccine cocktail that contains 9 immunogenic effectors which reduced the shedding of STEC O157 as efficiently as the Econiche® vaccine in a streptomycin treated mouse model. Although these results are promising for the development of a second generation

vaccine, the next step must involve the testing of this vaccine cocktail in large animals such as cattle.

In summary, this study has demonstrated that proteins such as Tir, EspB, EspD, NleA and EspA are highly immunogenic in both vaccinated and naturally infected subjects and as such could be potential candidates for a recombinant STEC vaccine. We have also shown that mice vaccinated with a cocktail vaccine containing 9 immunogenic effectors including Tir, EspB, EspD, NleA and EspA was capable of reducing shedding as effectively as the current STEC T3SPs vaccine, Econiche®.

7.0 GENERAL DISCUSSION AND CONCLUSIONS

7.1 General discussion

The goal of this study was to investigate the potential of developing a cross-protective vaccine against STEC serotypes. When the study began, no FDA approved vaccines which could protect against colonization or reduce shedding of STEC serotypes in cattle were available. Various candidate vaccines had been tested, however they appeared to be either serotype specific or had limited efficacy. The focus on reducing animal shedding is important as it is believed that this reduction would indirectly decrease the occurrence of human disease, which is most commonly transmitted zoonotically from animals such as cattle.

Potter and colleagues demonstrated that the vaccination with T3SPs was effective in the reduction of shedding of STEC O157 under both experimental and field conditions (Potter, Klashinsky et al. 2004). This vaccine, which is now called Econiche® (Bioniche Life Sciences) is based on T3SPs from STEC O157:H7, which are secreted and harvested from the supernatant of the bacterial culture. Since the homology among STEC serotypes of many of these proteins is over 90%, we hypothesized that Econiche® was a strong vaccine candidate to provide cross-protection against a number of virulent non-O157 STEC serotypes such as O26, O103 and O111.

Preliminary *in vitro* studies using immunogenic LEE-encoded proteins EspA and Tir, which are present in the current Econiche® vaccine and critical for the formation of the T3SS, were encouraging. Western blot analysis using polyclonal antibodies against Tir and EspA from STEC O157:H7 revealed a significant level of cross-reactivity against Tir and EspA proteins from non-O157 serotypes. These early studies were promising, which led us to pursue the possibility of cross-protection based on the Econiche® vaccine. However, more detailed studies using both ELISAs, as well as functional inhibition assays which are commonly used in STEC studies, demonstrated limited cross-reactivity amongst all STEC serotypes tested. Homologous sera raised against T3SPs proteins was capable of inhibiting STEC from attaching to HEp-2 cells, the same sera failed to inhibit the attachment of any other serotype suggesting that protection based on T3SPs, which is the basis of the Econiche® vaccine, will be serotype specific. This is in

agreement with other studies that have shown similar results. A subunit vaccine from *Streptococcus uberis* and *Streptococcus dysgalactiae* using proteins with 92% identity did not confer cross protection following immunization due to the presence of non-conserved regions that contained the protective epitopes (Fontaine, Perez-Casal et al. 2002). Therefore, it is perhaps not surprising that cross reactivity did not occur due to the significant variability of the T3SPs EspA and Tir, among the four serotypes tested.

Since our initial hypothesis was proven incorrect we decided to test the potential of a recombinant vaccine using a chimeric protein in order to achieve cross-protection against STEC serotypes. Chimeric proteins have been commonly used as vaccine components and have been successful in achieving protection and eliciting high titers. The target we focused on was the STEC O157:H7 Tir protein, since several studies have reported that Tir is highly immunogenic and capable of producing high antibody titers when used as an antigen for vaccination (Asper, Sekirov et al. 2007). Human sera collected from HUS patients shortly after the onset of an STEC infection reacted strongly with the Tir protein from STEC O157:H7 (Li, Frey et al. 2000). Potter and colleagues have also demonstrated that the vaccination of cattle with T3SPs harvested from a Δ*tir* STEC O157 strain did not reduce shedding as efficiently as T3SPs from a wildtype O157 strain (Potter, Klashinsky et al. 2004). These results demonstrate that a chimeric Tir protein had the potential to protection against heterologous serotypes.

To provide cross-protection we believed that the chimeric Tir protein would require the inclusion of immunogenic epitopes from non-O157 Tir proteins. The identification of peptides focused on the intimin binding domain as this region is the only part of the Tir protein which is exposed on the outside of the epithelial cell during infection. This region is required for the binding of intimin which allows for the formation of A/E lesions. Although Tir has a significant homology amongst STEC serotypes the pattern of immunogenic epitopes where very different amongst the different serotypes (Figure 5.2).

The use of the chimeric Tir protein as a vaccine did not result in a reduction in shedding of either STEC O157 or O111 in the streptomycin-treated mouse model. Interestingly similar results have also been seen with other LEE proteins such as EspA, EspB and intimin, as well as STEC colonization factors such as Efa1, where a strong

antibody titer was induced, but protection was not observed (Dziva, Vlisidou et al. 2007; van Diemen, Dziva et al. 2007). Based on these results we believe that the protection seen with the Econiche® vaccine could be a result of the cumulative effect of a number of immunogenic secreted proteins present in the bacterial supernatant used for vaccination.

In order to confirm this hypothesis, we cloned and expressed 66 LEE and non-LEE encoded genes followed by purification using the HIS-tagged method. The purified proteins were tested using sera raised against T3SPs from STEC O157 and non-O157 serotypes and a number of proteins which were not only highly immunogenic, but also cross-reactive were identified. These proteins have the potential to be incorporated into a cross-protective vaccine which would protect against a number of virulent serotypes such as O157, O26, O103 and O111.

Cattle are one of the most important animal reservoirs and are a major source of STEC infection of humans. Cattle infected with STEC do not show any clinical symptoms, unlike humans who are vulnerable to diseases such as HUS and TTP. In this study we also tested sera from both naturally infected humans and cattle to learn if the pattern of recognized proteins was different. Recognition by human and cattle sera followed a similar pattern, but while the cattle response against the purified proteins was fairly consistent, the magnitude of the response by the human HUS sera appears to differ. Although, humans and cattle show different disease symptoms, both hosts still appear to be colonized by STEC in a similar manner since both hosts recognized a similar pattern of virulence proteins.

Based on the identification of a number of highly immunogenic purified proteins where several appear to be cross-reactive, we used the streptomycin-treated mouse model, and found that a 9-effector cocktail, which contained the chimeric Tir protein, was capable of reducing shedding of STEC O157:H7 as efficiently as the Econiche® vaccine.

These results using the purified proteins were encouraging for the development of a second generation vaccine against STEC O157. To further confirm these results, it will be necessary to test the efficacy of this 9-effector cocktail in a larger animal model such as cattle. By testing in cattle we would be able to determine if this recombinant vaccine is truly as efficient as the Econiche® vaccine. A recombinant vaccine would be

advantageous, since production of the current vaccine is labour-intensive and costly to produce.

If the 9 effector recombinant vaccine provides protection against STEC O157 it will then be useful to test for cross-protection against heterologous serotypes in cattle. However, if the current vaccine does not protect against non-O157 serotypes, there is also the possibility of the development of a third generation vaccine where each effector could be further developed into a chimeric protein using the same method as used for the construction of the Chimeric Tir protein. However this would involve the sequencing of all used effectors for the non-O157 serotypes (O26, O103 and O111) for which the whole genome has not yet been made publicly available.

Our overall goal was to develop a vaccine to protect against a number of virulent STEC serotypes. Although our original hypothesis was not confirmed, we have made significant progress in identifying possible candidates which can be used in a cross-protective vaccine. As well, we have shown that by using synthetic peptides, we can construct chimeric proteins which contain multiple immunogenic epitopes that, when used as a vaccination antigen, each individual epitope can be recognized by a host. This knowledge along with the newly identified immunogenic and cross-reactive effectors has built a platform for the production of a STEC cross-protective vaccine.

7.2 General conclusions

- Protection of cattle based on T3SPs appears to be serotype specific.

- A chimeric Tir protein was not protective against challenge by STEC O157 and O111, which contanined unique epitopes identified in the intimin binding domain of the Tir protein.

- We have identified a number of immunogenic and cross-reactive LEE and non-LEE encoded effectors.

- After natural infection both humas and cattle sera appear to recognize similar pattern of STEC T3SPs even though both produce very different symptoms to the infection.

- A cocktail vaccine containing 9 STEC effectors was capable of reducing shedding as effectively as the current STEC T3SPs vaccine Econiche® in a streptomycin-treated mouse model.

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